Vol. 90 Part 4 Dec. 1949

The Quarterly Journal of Microscopical Science

(Third Series, No. 12)

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LONDON: GEOFFREY CUMBERLEGE

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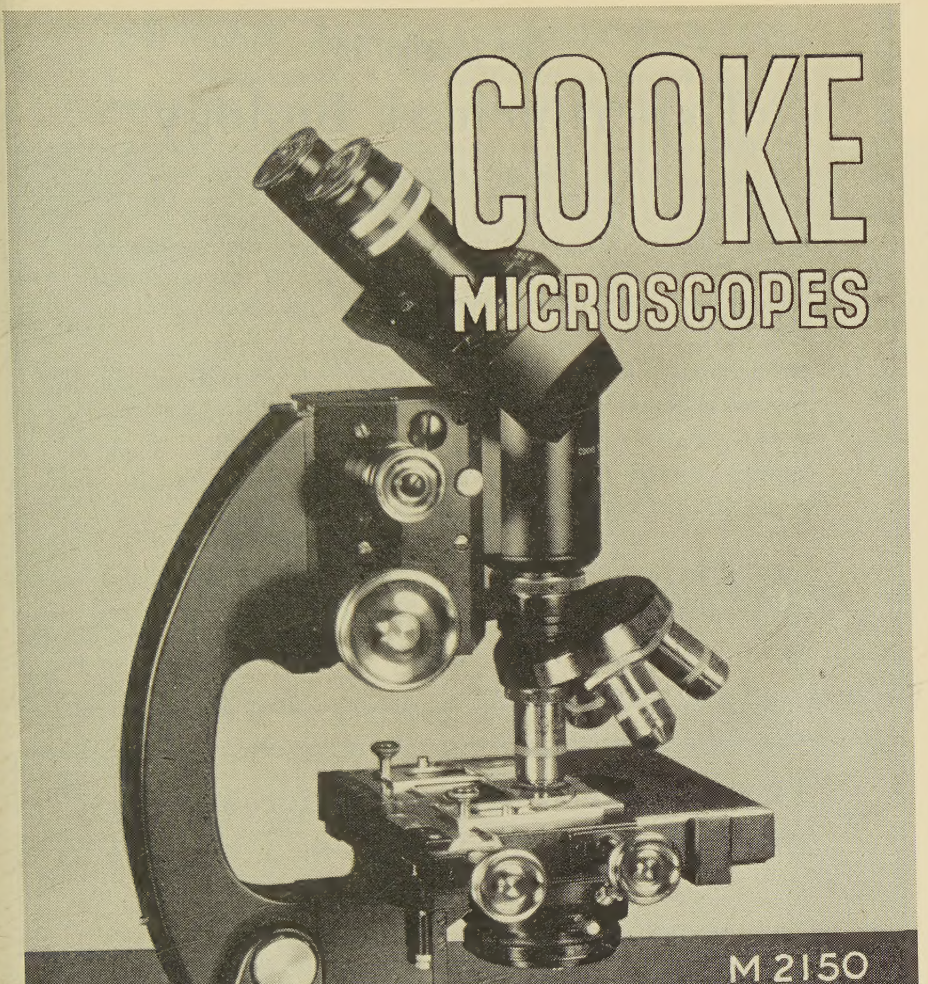
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¶ The current issue is Volume 26, No. 3, October 1949.

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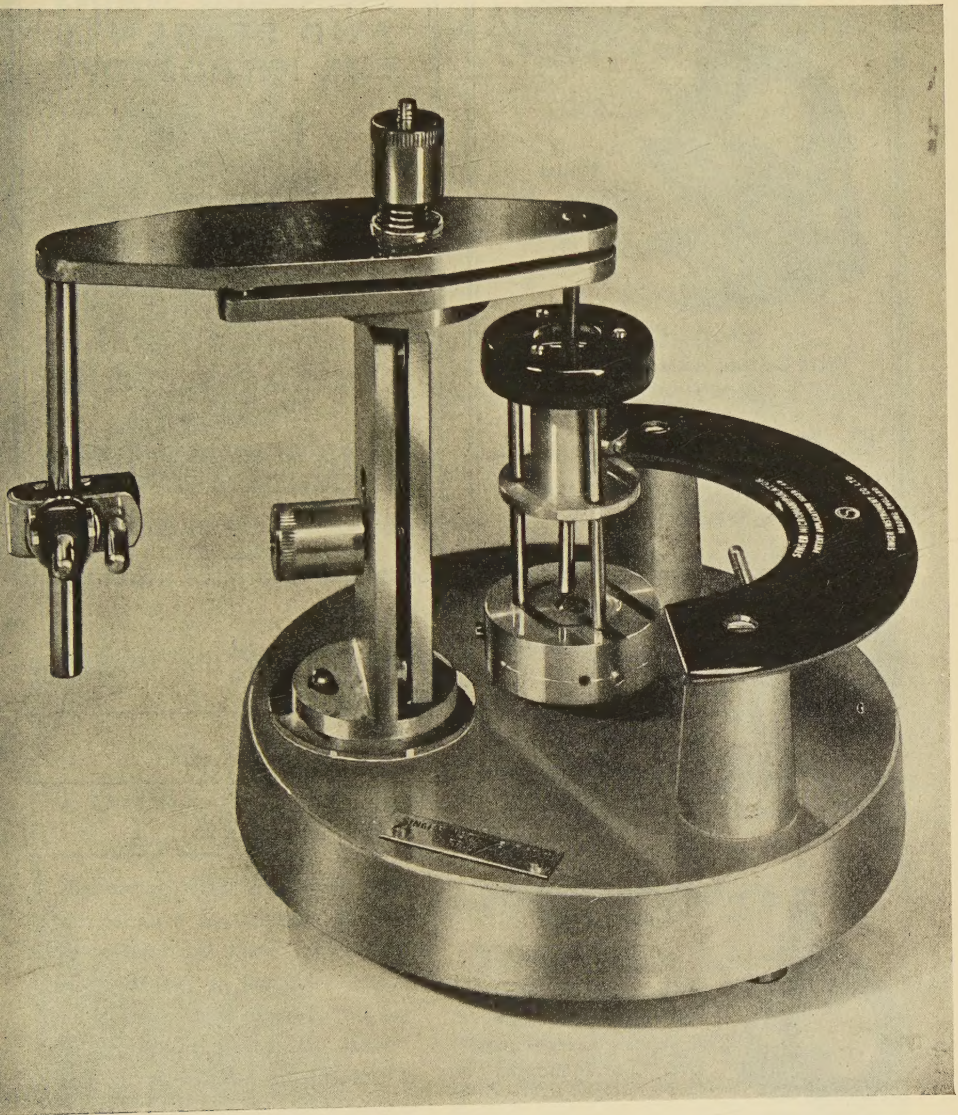

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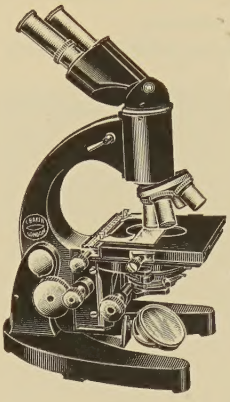

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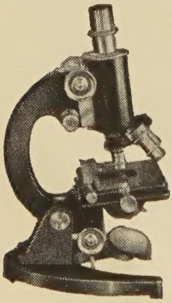

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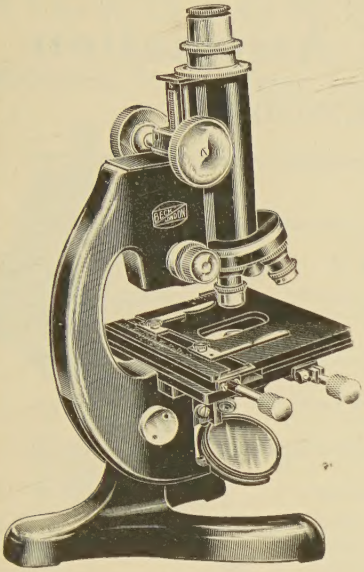

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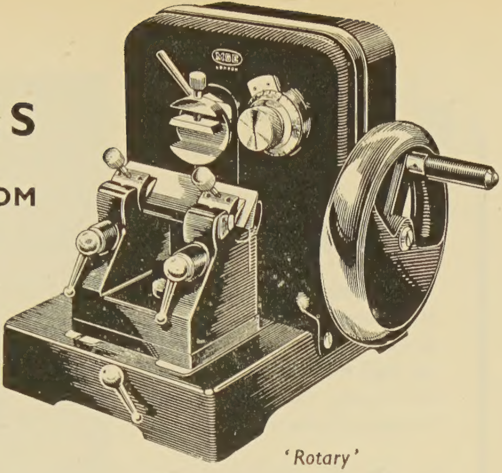
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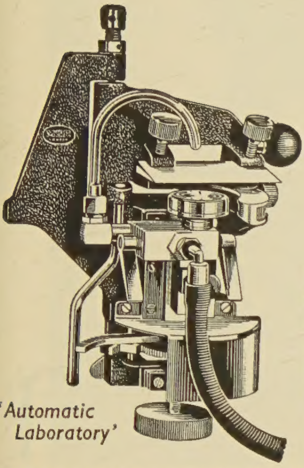
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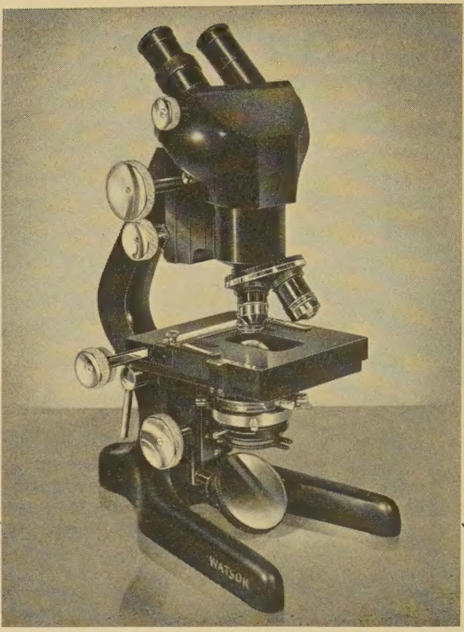

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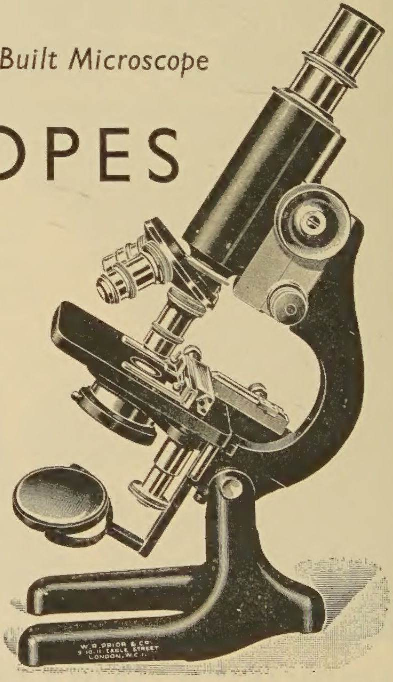
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On the Functional Morphology of the Alimentary Tract of Some Fish in Relation to Differences in their Feeding Habits:

Cytology and Physiology

BY

A. H. AL-HUSSAINI, M.Sc. (Cairo); Ph.D. (Sheffield)

(*From the Department of Zoology, the University of Sheffield, England*)

With four Plates

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HISTORICAL INTRODUCTION

THE present paper forms the continuation of work already reported in an earlier paper (Al-Hussaini, 1949). The same three cyprinid fishes have been used for comparative study, namely, the mirror carp, *Cyprinus carpio* as an example of a herbivore, the roach, *Rutilus rutilus* as an omnivore, and the gudgeon, *Gobio gobio*, a predominantly carnivorous fish.

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

Much of the general literature concerning the alimentary canal of fishes has been reviewed in Part I of this work and only those papers directly bearing on the subject under review will be mentioned. Amongst the earliest workers to investigate the physiological aspects of the fish gut were Tiedmann and Gmelin (1827), while half a century later Homburger (1877) studied digestion in several species of carp, and reported that the bile and extracts of the liver as well as of the intestinal mucosa were capable of digesting fibrin, emulsifying fats, and of hydrolysing starch to sugar. Krukenberg (1877–8) concluded that, although no fish possesses salivary glands, some, as *Cyprinus carpio*, have a diastase in the mucous membrane of the mouth, while in some teleosts, such as *Cyprinus* and *Gobius*, the organ occupying the position of the stomach secretes no enzymes at all, digestion being effected in the middle intestine. He found trypsin in this region as well as in the liver in cyprinids. In contrast to Krukenberg, Luckhau (1878) found no enzyme which would digest albumen. He also failed to find a lipase or any proteolytic enzyme working in an acid medium (pepsin). He did, however, find an amylase and a proteinase working in a neutral or alkaline medium (trypsin) in various parts of the intestine. Blanchard (1882) found an amylase and a lipase in the rectal gland of elasmobranchs and an amylase and a proteinase in the pyloric caeca of teleosts, which structures he regarded as representing a pancreas. The latter finding was confirmed by Stirling (1884) working on the herring, cod, and hake.

Kenyon (1925) studied the digestion of carbohydrates and proteins in cold-blooded vertebrates comparatively, and contrasted the processes with those of a mammal (dog). Amongst his examples of teleosts he used the common carp. He concluded that little change in the general character or rate of activity of enzymes has occurred during the evolution of vertebrates but that a close correlation exists between the morphology of the gut and the distribution of enzymes (e.g. no pepsin in the stomachless carp). He demonstrated erepsin for the first time and showed certain adaptive features of enzymes to the diet (e.g. more amylase in herbivorous than in carnivorous species). Vonk (1927) studied the range of temperature and pH over which fish enzymes (including those of the common carp) are active, and their respective optima. He found the digestion of fishes similar in most respects to that of mammals. In 1937 he reviewed the specificity and collaboration of digestive enzymes in the Metazoa.

The more specialized cytological and histochemical literature is very recent and it will be more convenient to deal with this at the relevant places in the text, as there are very few previous observations on fishes and most of the references involve comparative discussion.

MATERIAL AND METHODS

The material used in the present study came from the same sources as that used for the anatomical and histological study (cf. Part I). Numerous techniques were used, many of them standard procedures, but some required special modification. It will, therefore, be more convenient to refer to such technical methods in the appropriate sections of the text.

I. The Cytology of the Intestinal Epithelium

A. *The Absorptive Cells*

1. *The Free Border*

Baker (1942) based his study of the free border of the intestinal absorptive cells of vertebrates on tetrapods and made no mention of fishes. In a personal communication to the writer he said that he examined two fresh-water fishes only, and that their free border was simpler in structure than that of his tetrapod types. Baker's methods have therefore been applied to the present three cyprinids with the object of discovering any possible deviation from the general plan which he described as common to all the tetrapods he had examined. The crested newt, *Triturus palustris*, was used as a control. Material from the three Red Sea fishes studied earlier (Al-Hussaini, 1945, 1946, 1947) was also reinvestigated from this point of view.

For a full history of the literature dealing with the luminal edge of the absorptive cells of vertebrates reference should be made to Baker's review, but, briefly, it may be said that there are two theories; the one claims that the border consists of cilia or rods, and the other that the border is traversed by minute canals which open at the surface as pores. Baker adheres to the second alternative (cf. Baker's text-figs. 1 and 2).

In the cyprinid absorptive cell the free border is built up of three principal layers (Pl. I, fig. 1). These are the superficial layer (SF.L.), the canal layer (C.L.), and the granular layer (GR.L.). Immediately deep to the free border, within the cytoplasm of the cell, is the subgranular layer (SG.L.), and this, in turn, is separated from the supranuclear group of mitochondria (SP.M.) by a clear zone (CL.Z.).

The free-border in the cyprinids is rather thinner (about $2{\cdot}5\,\mu$) than that of the newt. The superficial layer is best seen in living cells examined in isotonic saline, or in iodized serum, or in preparations fixed and mounted in 4 per cent. formaldehyde; in other words, in aqueous mounted material. It appears as a very thin strip extending along the entire surface of the free border, which, on careful focusing, is seen to display a beaded appearance rather than a uniform thickness; nevertheless very few openings of the canals were encountered. In clarite mounted material the superficial layer is usually obscured, but in two instances it was seen in material (from *Rutilus* and *Cyprinus*) fixed in M.A.S. of Baker and stained with Heidenhain's haematoxylin.

The canal layer can be demonstrated by several techniques, ordinary routine ones as well as those intended for special purposes, e.g. Kull's technique for mitochondria, or Ciaccio's for lipines (Pl. I, fig. 2), although Baker claims that fixation in M.A.S. followed by acid violet yields the best result with his material. The canals may even be seen in the living cell as faint striations. The canal layer stains light green with Masson's trichrome stain, indicating, as Baker suggested, that it contains collagen. The superficial layer stains an even more intense green by this method. The canals usually appear spindle-shaped, although sometimes they appear constricted in the middle; they are narrower and fewer in number than in the newt.

It is uncertain whether the structures revealed by routine methods are the canals themselves or optical sections of the substance between the canals. The striking appearance of the canal layer has resulted in some reference to the free border appearing in almost every work on the histology of the fish gut, although it is described under various names, e.g. 'striated border', 'brush border', 'top plate', or 'cuticle'. Some writers have described cilia in various places in the intestinal epithelium of teleosts, such as at the entrance to the pyloric caeca (Edinger, 1877), within the pyloric caeca (Rahimullah, 1945), or along a very short tract of the long intestine of *Salarius enosimae* (Ishida, 1935). It is possible that the structures described as cilia are really the inter-canal substance in a border unusually thick.

At many places between two adjacent cells there is a canal which, while still fine, is larger than the canals just described and which opens freely to the surface by a distinct pore. Baker considers this canal to be an artifact due to an interruption of the border and not a distinct passage; nevertheless, its frequent and persistent occurrence makes one sceptical about its artificial nature and I am more inclined to regard it as a real structure offering permanent passage into the cell, although one must admit that it cannot be found at *every* cell junction.

The granular layer appears as a dark line in the living cell but shows no granulation, and this may also be true in the fixed cell when treated by some methods, e.g. by Baker's modification of the Smith–Dietrich method for lipines, or with Schulze's chlor-zinc-iodine, although in the latter case the dark line shows a slight intermission. If the sections are given a preliminary treatment with diaphanol before applying Schulze's method, the granular layer becomes somewhat swollen, but no spherical granules such as those described by Baker could be seen.

The subgranular layer and the clear zone are distinct both in the living cell and in material fixed in M.A.S. In sections specifically prepared to demonstrate mitochondria the clear zone is especially distinct.

The intercellular band (Pl. I, fig. 1, I.B) may be easily observed in the living cell and stands out clearly in material stained by Heidenhain's haematoxylin (Pl. I, figs. 3 and 5), while with mitochondrial techniques he band takes the same colour as the mitochondria and the granular layer. It is in the form of a rim or girdle which encircles the edges of the cells approximately at the level of the granular layer (its precise position may be seen in Pl. I, fig. 1) so that in tangential sections the band appears as a polyhedral or hexagonal outline like a honeycomb (Pl. I, fig. 3). Its deep edge merges imperceptibly into the intercellular membrane. Baker described the band in tetrapods as 'usually wider than high', but in the cyprinid gut its dimensions are reversed.

When two limbs of adjacent folds come into direct contact with each other the border appears much thicker than usual and the canals appear stretched (Pl. I, fig. 4), suggesting that the free border is elastic and capable of expansion and that this has been effected by the temporary adhesion of the free surfaces, so that, when the muscularis layer contracts during the normal peristaltic movement of the intestine, the free border is slightly stretched. On the other hand, the reverse may occur, and the free border may appear in places to be very much contracted so that none of the details just described may be seen. It follows therefore that the border may, and probably does, undergo a series of changes during the absorptive processes, and it is by no means impossible or even improbable that these changes may assist the passage of digested food into the cell.

In *Scarus sordidus*, the free border is of the 'brush border' type. In the anterior unsacculated portion of the intestine (duodenum) the border is particularly deep (4·5 μ against 2–3 μ for the remainder of the gut) and the canals are sometimes constricted in the middle and sometimes widely separated. The passages between adjacent cells are clearly seen and the border appears to consist of 'clutches' of canals, each 'clutch' belonging to one absorptive cell (Pl. I, fig. 7). In *Mulloides auriflamma* and *Atherina forskali* the free border is similar to that of the cyprinids.

A species of chicken-fish, *Pterois volitans*, has a peculiar free border. It is strikingly thick (7–11 μ) and along the bases of the crypts, where the absorptive cells are shortest, the border accounts for about one-third of the total length of the cells. It appears as though consisting of interlacing fibres leaving between them distinct passages, sometimes of considerable calibre, presumably equivalent to the canals of other fishes (Pl. I, fig. 6).

The free borders described here can be arranged in a series which might perhaps have an evolutionary significance, showing the transition from the ciliated epithelium of lower chordates to the typical free border described by Baker in tetrapods. In such a series *Pterois* would be the most primitive, the duodenal epithelium of *Scarus* intermediate, and the remaining species the most specialized.

2. *The Mithochondria*

Numerous methods were employed for this section of the work, but it was found that Kull's method following fixation in Helly's fluid and post-chroming at 37° C. for 2 days (cf. Volkonsky, 1928, and Baker, 1945) was superior to all the rest.

The mitochondria are arranged in two groups, as in mammals; both contain rod-like as well as granular chondriomes (Pl. I, fig. 1), but the rod-like form is commoner in the supranuclear group and the granular type is more frequent in the subnuclear zone which extends into the basal processes of the cells and is better developed in *Gobio* than in *Cyprinus* or *Rutilus*. Indeed, the mitochondria are on the whole coarser in the carnivorous gudgeon than in the other two fish, but this difference may well be due to specific causes rather than to the nature of the fish's food, since these organoids are subject to great variation in different cells even of the same fish.

Concerning the function of the mitochondria in the intestinal epithelium, Champy (1911–12) found that, in the mammal, during digestion and absorption the mitochondria in the cells of the intestinal villi become thread-like in form and less numerous. He suggested that their bipolar distribution is associated with the dual function of secretion and absorption. Liu (1930) working with mice found that the intestinal mitochondria show no fusion or decrease in number during carbohydrate absorption, but that during fat absorption they change rapidly within the first 6 hours after feeding the animal on fat from the rod-like form to fused masses. Cowdry (1924) also found a progressive decrease in mitochondrial numbers with an increase in the number of fat globules, as did Bourne (1942). Duthie (1935) showed that surviving liver cells, soon after transplantation, often store fat in large quantities and that during this process the mitochondria become short rods and lie in close relation to the fat droplets, but, on the other hand, Cramer and Ludford (1925) maintain that mitochondria do not appear to take any active part in fat absorption.

Experiments with the present cyprinids have shown that 3 days after an animal diet, while fat absorption is still going on but when the gut lumen is free from undigested food, the mitochondria of the absorptive cells are less numerous than they are when the gut is resting. The globules of absorbed fat may be found scattered throughout each absorptive cell except in the clear zone and for a short distance below it; those lying in the luminal portion of the cell being larger than those towards the base. Each fat globule appears red-edged which, on critical examination under oil immersion, is seen to be due to the acid fuchsin taken up by the mitochondria, thus suggesting that these organoids do have a close association with fat absorption. Confirmatory evidence was obtained by giving a small fish (about 10 cm. long) a meal rich in fats through the mouth and killing it 4 hours later. Abundant fat globules had already passed into many absorptive cells but not into all of them, thus making it very easy to compare the disposition of mitochondria in active with that in inactive cells within the same fish. The active cells showed a picture similar to those of the fish killed 3 days after feeding. The mitochondria had already undergone a rapid disintegration and lost much of their rod-like shape. The inactive cells which had not absorbed any fat resembled those of a fasting fish. In a second experiment four fasting roach of about equal size were each given a meal in semi-fluid form of different composition. The first was a suspension of starch, the second one of casein, the third a fat emulsion, and the fourth an inert substance (magnesium trisilicate in liquid paraffin). The fish were killed 4 hours afterwards. Subsequent examination showed practically no change from the resting condition in the mitochondria of the absorbing cells of the first and fourth fish, those of the second fish showed only a slight diminution in their numbers, but the third fish gave a picture similar to that of the fat-fed fish described above. The close association between mitochondria and fat absorption in fishes, as in mammals, seems therefore to be well substantiated.

3. *The Golgi Element*

Baker (1944), in his valuable contribution to our knowledge of the structure and chemical composition of the Golgi element, concluded that it consists essentially of four parts, namely, 'neutral red vacuoles', 'dense lipoid-containing substance', 'diffuse lipoid-containing substance', and 'Golgi product' (cf. Baker's figures on pp. 24–5 of his paper). He used various types of cells for his study, including the absorptive cells from the intestine of the newt. Baker contends that the classical methods used to produce the Golgi network cannot be relied on to give an accurate picture of the structure of the element during life, and accordingly he introduced a new procedure—the formol-Sudan-black technique. In the present study this new technique, several osmium and silver impregnation methods, as well as intra-vitam stains were used (*vide infra*).

When the intestinal epithelium is examined in the living condition in saline in a compressorium the vacuoles of the Golgi element are clearly seen, and are readily stainable intra-vitam by neutral-red as described by Baker or by methylene blue by the method of Worley (1943). The majority are spherical but are variable in size from cell to cell or even in the same cell and their arrangement follows no definite pattern. Some three to five vacuoles are found in each cell.

In fixed preparations the Golgi element always lies in the apical part of the cell on the luminal side of the nucleus. Corti (quoted from Macklin and Macklin, 1932) maintains that in the gastric glands of the frog and mouse it may be either supra- or subnuclear, but it has never been seen in a subnuclear position in the cells of the cyprinid gut. It may be separated from the nucleus by a short interval or it may abut closely on it to form a sort of cap (Pl. I, fig. 1, G.E). With Baker's formol-Sudan-black technique a few of the cells showed the Golgi element clearly, but in the majority it appeared only as a dark area. The vacuoles were sometimes visible and in addition thread-like structures could be detected. They lie more or less parallel with the longitudinal axis of the cell, but are not quite straight. Incidentally, it should be mentioned that both groups of mitochondria and the granular layer of the free border of the absorptive cells as well as the intercellular band were all stained blue-black by this technique but less intensely than the Golgi element.

The dense lipoid-containing substance is impregnated strongly by Kolatchev's method and appears as two or three black threads enclosing a number of vacuoles, varying in size and number, and hardly separated by any intermediate substance (Pl. II, fig. 9) and without showing the Golgi product (cf. Baker, 1944). A comparable result is obtained by Weigel's technique and also by Nassonow's method except that in the latter case the dense lipoid-containing substance is more compact, the whole element appears contracted, and, except in a few cells, the vacuoles are not so distinct. The osmium impregnation method which best preserves the vacuoles is probably Baker's formol-osmium technique. With this method the Golgi element appears pale and more 'vacuolated' than with any of the other methods, but the dense lipoid-containing substance is not so heavily osmified.

The Golgi element requires a longer period for osmification in *Rutilus* and *Cyprinus* than in *Gobio*, but it is difficult to see any connexion between this and the nature of the food.

Two silver impregnation methods (Da Fano and Aoyama) were tried but with little success. A faint impregnation of the dense lipoid-containing substance occurred in one case only (intestine of *Gobio*) and that by Aoyama's method. Baker experienced a similar difficulty with material from the newt. This surprising result cannot be explained.

If the absorptive epithelium be examined in the active state a noticeably different picture is obtained. If material from a fish which has recently fed on food rich in fats be examined, fat globules will be so abundant in some cells as to mask the Golgi element completely; nevertheless others, which have not absorbed much fat, still show little of the Golgi element. In osmicated material the dense lipoid-containing substance appears greatly diminished and no vacuoles of any shape can be seen; nevertheless, in intra-vitam neutral-red preparations vacuoles show up clearly and are seen to be nearer to the free border than is the case in the resting cell. It would thus appear that the vacuoles are normally confined or held in place by the dense lipoid-containing substance which disintegrates almost completely during the process of fat absorption. Thus the Golgi element, as well as the mitochondria, is apparently involved in this process.

B. *The Goblet Cells*

Much of the extensive literature on goblet cells has already been reviewed by Oppel (1896) and by Macklin and Macklin (1932), but there are still some discrepancies in the accounts even of such well-known structures as goblet cells. For example, it is not known whether goblet cells are developed from undifferentiated columnar cells, from modified absorptive cells, or whether they are formed *sui generis*. Again it is uncertain whether goblet cells discharge their contents once and for all, or whether this is a continuous process.

The mucous contents of the cells have been stained by the following methods: Mayer's mucicarmine; Lillie's toluidine blue (Cowdry, 1943); toluidine blue of Hempelmann (1940); periodic acid followed by Schiff's reagent (McManus, 1947), while the zymogen granules were stained either by Bensley's neutral gentian or Bowie's ethyl-violet-Biebrich-scarlet (Cowdry, 1943).

The stoma of the goblet cell is noticeably widened when mucus is being discharged. According to Macklin and Macklin (1932) the luminal end of the goblet cell is thought to be encircled by the intercellular band ('terminal bars') and to be covered by a 'top-plate' which is ruptured to form a stoma when the contents are being discharged. The present observations do not agree with this. By careful focusing up and down on vertical sections the free border may be seen to be continuous above and below the stoma; thus the stoma is a permanent opening which widens or narrows according to whether mucus is being discharged or not; evidence that the free border is elastic has already been given, p. 327. The stoma of the goblet cell may therefore be regarded as possibly of a similar nature to a large canal of the canal layer of the absorptive cells, or to the passage between two adjacent absorptive cells.

The contents of the goblet are produced in the form of granules which stream out as a shapeless mass. According to Duthie (1933) fixation with Regaud followed by staining with Bensley-Cowdry's method for mitochondria gives a red colour to premucin (mucigen) and a greenish-blue colour to the formed mucus. Applying this technique to the cyprinid gut, goblet cells showing either premucin or mucus may be found scattered haphazardly throughout the folds. Experimental evidence, which will be discussed in fuller detail in a subsequent paper, has been obtained which suggests that the cells of the intestinal epithelium, both goblet and absorptive cells, are regenerated at the bases of the crypts and that they migrate along the sides of the folds to the crests before becoming effete. If this view is correct it is unlikely that the goblet cell discharges its contents once and for all since all stages of mucus formation may be found anywhere on the folds.

The goblet cells of the entire intestine produce zymogen granules. These granules stain more intensely in *Gobio* than they do in *Rutilus* or *Cyprinus*, suggesting that zymogen has a higher concentration in the carnivorous species. Differentially, the highest concentration of zymogen occurs in the rectum and the third limb of the intestine, the stain in the intestinal swelling being quite pale, thus suggesting that protein digestion by enteric proteinases is largely carried out in the posterior segments of the intestine. A further differential effect may be noted, namely, that the granules in the cells at the apices of the folds do not usually stain so intensely as those at the sides but take only a very pale colour with zymogen stains, suggesting that the goblet cell loses some of its potentiality to produce zymin as it becomes senile while still retaining its capacity to produce mucigen.

The mitochondria of the goblet cell (Pl. I, fig. 1) lie in its basal portion. The Golgi element (Pl. I, fig. 1; Pl. II, fig. 9) is better developed than that of the absorptive cell, while its vacuoles are very variable in size and some of them are far from spherical.

Certain modifications of the goblet cell which appear to give rise to a 'pear-shaped' cell (Pl. I, fig. 1, P.C.R., P.C.G.) will be described in a subsequent communication.

C. *The Granular Cells*

So extensive is the literature dealing with the granulocytes that occur in various vertebrate classes and so controversial and conflicting are the views of the various authors concerning their appearance, nature, and function (cf. Michels, 1923; Bolton, 1933), that it is not easy to discuss them briefly. However, two important conclusions can be reached from a survey of this literature, namely, that the cells are present in some species but not in others (Michels) and that, when present, they may be either basiphilic (mastcells or basiphils) or acidophilic (acidophils), while some authors (Michels, 1923; Bolton, 1933; Duthie, 1939) have described hybrid cells showing both basiphilic and acidophilic granules. Bolton claimed that the staining reaction may differ as a result of impurities or variations in the constituents of different batches of dye, while Duthie advises the use of several stains, a single stain being unreliable.

In view of this unsatisfactory state of affairs a revision of previous work is needed and much research, both cytological and experimental, is required before we may fully understand the nature of these cells, and it is not intended, in the present limited study, to devote undue length to this wide problem. An endeavour has been made, nevertheless, to make some contribution to our knowledge of the structure of the granular cells and their function as they occur in gut of fishes. The material studied, in addition to the three cyprinids with which we are especially concerned, includes *Scarus sordidus*, *Mulloides auriflamma*, and *Atherina forskali* from the Red Sea, *Crenilabrus melops* and *Trigla hirundo* from Plymouth, and *Salmo trutta* from various localities in England. In several cases sections from different species were passed simultaneously through the stains, hence excluding the possibility that differences might be due to impurities or differences in the dye.

The following techniques were employed and the results obtained are tabulated in Table 1:

1. Fixation in Bouin's fluid, stained in haematoxylin and eosin. This was the routine method applied to the Red Sea types in earlier work (Al-Hussaini, 1945, 1946, 1947).
2. Fixation in Zenker-formol, stained with Giemsa (as employed by Duthie).
3. Fixation in Zenker-formol, or Helly's fluid, stained with neutral-gentian of Bensley (as employed by Bolton).
4. Fixation in Helly's fluid, stained with ethyl-violet-Biebrich-scarlet (also employed by Bolton).
5. Fixation in absolute alcohol, stained with thionin (saturated solution in 80 per cent. alcohol) (as used by Michels).
6. Mitochondrial techniques—either Bensley-Cowdry's or Kull's method. So far as is known these techniques have not been previously employed in the study of granular cells.
7. Demonstration of glycogen by Best's carmine method.

A few other methods were applied to the granulocytes of the three cyprinids only; these will be referred to below.

TABLE 1. *Reaction of Granular Cells to Certain Fixatives and Stains in Some Teleostean Species*

Fish	*Techniques as in text* 1	2	3	4	5	6	7
Rutilus rutilus	Poor; cytoplasm pale pink.	Fair; granules reddish.	Very pale blue almost negative.	Reddish granules.	Poor; few cells show blue granules.	Good; granules red.	Bright red granules.
Gobio gobio	(*Granular cells absent*)						
Cyprinus carpio	As in *Rutilus.*	Very few cells show red granules, some bluish.	Pale blue granules.	As in *Rutilus.*	Few cells show bluish granules.	As in *Rutilus.*	As in *Rutilus.*
Scarus sordidus	Very good; red granules.	Very good; red granules.	Good; few cells show blue granules.	Some cells show red, others purple granules.	Negative.	,,	Negative.
Mulloides auriflamma	,,	,,	,,	,,	,,	,,	,,
Atherina forskali	Very good; bright red granules.	,,	,,	,,	,,	,,	,,
Crenilabrus melops	As in *Rutilus.*	,,	,,	Good, red granules.	As in *Cyprinus.*	,,	,,
Trigla hirundo	Very good; bluish-black granules.	Very good; blue granules.	Blue granules.	Purple granules.	Very few cells show pale granules.	Good; blue granules.	,,
Salmo trutta	As in *Rutilus.*	,,	Intense blue	,,	Pinkish granules.	Good; some cells show blue, others red, granules.	,,

It will be seen from the table that the granular cells of the fish gut may be grouped into two main categories: (1) Those which take a blue colour with Giemsa, or the basic component of the mitochondrial stains. These are basiphils and occur only in *Trigla hirundo* and *Salmo trutta.* (2) Those which take a red colour with Giemsa or the acid component of the mitochondrial stains. These are acidophils and are found in all the remaining species where granulocytes occur.

The fact that eosin does not always stain the granules (in *Trigla* they stain bluish-black with haematoxylin-eosin) supports the suggestion made in an earlier paper (Al-Hussaini, 1947) that the granulocytes are not true eosinophils. Jordan and Speidel (1924*a*) were evidently of this opinion since they describe granulocytes in various teleosts as pseudo-eosinophils. On the other hand, Gulland (1898) described the granular cells in the alimentary canal of the Atlantic salmon as eosinophils, as did Greene (1912) those in the king salmon. More recently Bolton (1933) has called the granulocytes of the sockeye salmon and some other members of the Salmonidae as basiphilic, thus agreeing with the present observations on *Salmo trutta.*

In agreement with the results shown in Table 1, Duthie (1939) found that the basiphilic granules of *Trigla* were not well fixed by alcohol alone (column

5), though when mercuric chloride is added (e.g. Zenker-formol, column 2) perfect fixation is obtained. On the other hand, Bouin's fluid, though without mercuric chloride, fixed the granular cells of *Trigla hirundo* well (column 1). Bouin's fluid also proved satisfactory for the acidophils of *Scarus*, *Mulloides*, and *Atherina*. Preparations 6 years old, mounted in balsam, are still keeping very well (Pl. II, fig. 10). In contrast to this, the granulocytes of *Rutilus* (Pl. II, fig. 11), *Cyprinus*, *Crenilabrus*, and *Salmo* are poorly preserved by Bouin's fluid. Michels (1923) did not find Helly's fixative so satisfactory for preserving the granules in the 'mast cells' of *Leuciscus* sp. (allied to the roach) as those in *Cyprinus carpio*. He accordingly abandoned aqueous fixatives in the belief that the granules are rapidly dissolved by them and used absolute alcohol, but, as shown in Table 1, column 5, this would appear to be the least reliable of all the fixatives used in the present work. His work is criticized by Jordan (1926) who claimed that the so-called 'mast cells' of *Leuciscus* sp. must be acidophils and thought that the metachromatic staining of the granules by thionin as described by Michels must have been due to impurities in the dye. Duthie (1939), however, repeated Michels's work on the common carp and found that the granulocytes in the intestine are identical with those of the blood and other tissues, and that they all give basiphilic reactions. As may be seen from Table 1, the results of the present investigation show that only a few granules are preserved by alcohol and they stain orthochromatically (i.e. blue) in *Rutilus*, *Cyprinus*, and *Crenilabrus*. The reaction is especially strong in *Rutilus* and, although the granules stain less intensely in *Cyprinus* and *Crenilabrus*, they are far from being reddish. These results, therefore, agree with those of Jordan rather than with Michels or Duthie.

A further complication is introduced by the view put forward by Duthie and several other authors (cf. Duthie, 1939) that orthochromatic (or basiphilic) staining precedes metachromatic (or acidophilic) during the life of a cell. Jordan and Speidel (1924*a*, *b*) and Jordan (1926) also claimed that a progressive increase in the acidophilic quality of the granules is part of a ripening process. On this basis Duthie considers that the maturation of the coarse granulocytes is rapid in the Labridae but slow in the Triglidae. It will be seen that the Salmonidae resemble the Triglidae in this respect. Duthie, however, is inclined 'not to ascribe much importance to a colour change in the granules since it is probably influenced by physico-chemical factors in the surrounding tissues'. The present investigation can make no contribution to this aspect of the problem, but it is worth noting that in *Mulloides auriflamma* and *Salmo trutta* some of the granular cells exhibit both types of granules, acidophilic and basiphilic, while others behave differently according to whether they are outside or inside the intestinal epithelium. In *Trigla hirundo* the granular cells invading the epithelium remain entirely basiphilic even though they may migrate right up to the free border, but in *Atherina* and *Scarus* they remain acidophilic under similar conditions.

It is obviously impossible to draw general conclusions from the above data, and the picture is still further complicated by the fact that the number of granules which are stainable by one technique may be quite different from that seen with a different one, even in one and the same fish. For example, with mitochondrial techniques (No. 6) numerous granules are stainable in the granulocytes of *Cyprinus*, but only a small number are visible with techniques 2, 3, or 4. This suggests that even in one species the granules may be of differing chemical composition and hence may have several functions to perform.

Drzewina (1911) noted that the granulocytes disappeared from the blood and alimentary tract of certain Labridae during fasting but returned and were maintained in these sites during feeding. I have also noted a similar behaviour in granulocyte activity in *Mulloides auriflamma* (Al-Hussaini, 1946), but in *Rutilus* and *Cyprinus* their distribution is not connected with the presence or absence of food in the gut and most other authors make no mention of any such relationship. In order to try to discover more precisely the nature of the stimulus which evokes the migration of the granular cells into the intestinal epithelium of *Mulloides*, four specimens of about equal weight were kept without food for 3 days, and then on the fourth were given each an equal quantity of a semifluid diet of approximately the same consistency, but of different food content. The food was given through the mouth with a pipette and great care was taken to apply as nearly as possible the same pressure in each case. The first fish was given a suspension of casein, the second one of starch the third an emulsion of castor oil, and the fourth an inert substance (magnesium trisilicate in liquid paraffin). They were killed 4 hours later and sections of the intestine were prepared. Granular cells were present in equal profusion in the stomach wall of all four fish; hence since each kind of food was equally effective in producing a response as also was the inert magnesium trisilicate and liquid paraffin, the stimulus would thus appear to be due rather to the mere physical presence of material in the lumen of the gut than to its chemical nature or nutritional role. Perhaps the increased peristalsis occasioned thereby may play a part.

Some evidence of a wandering tendency in the granulocytes was observed in all the species examined. In *Rutilus* and *Cyprinus* the cells have never been observed to migrate farther towards the lumen than the basal third of the absorptive cells, whereas in the other fishes examined they migrate as far as the free border and in the majority they are ruptured as though discharging their granules. They are, of course, entirely absent from *Gobio*. *Rutilus* shows an interesting condition which appears to be so far unrecorded, in that granular cells are encountered in the pulp cavities of the pharyngeal teeth where they may be seen within the capillaries, alongside erythrocytes, as well as within the surrounding connective tissue. They were, however, never seen in the large blood-vessels, neither could they be demonstrated in blood films prepared by the usual routine methods. This, however, may well be due, as Drury (1915) suggested in a similar instance, to the instability of the granules.

The granular cells of *Rutilus* and *Cyprinus* show a positive reaction to the histochemical test for alkaline phosphatase (Pl. III, fig. 13), *Rutilus* showing a stronger reaction than *Cyprinus*. They also store glycogen (cf. Table 1, column 7).

Summaries of the ideas of various authors concerning the possible functions of the granular cells have been given by Bolton (1933) and by Duthie (1939), while I have also made a brief reference to the subject in an earlier paper (Al-Hussaini, 1946). They are usually credited with a secretory function of some sort, either exocrine or endocrine, and may possibly be concerned with lipase activity and or the elaboration of zymogen. Certainly the granular cells of *Salmo* and *Trigla* give a positive colour test for zymogen as, in a less degree, do those of *Scarus*, *Mulloides*, *Atherina*, *Crenilabrus*, *Rutilus*, and *Cyprinus*. Jordan and Speidel (1924*b*), on the other hand, believe that the 'intestinal eosinophils' (granular cells) may relate to the general immunity of the mucosa to the bacterial content of the gut by the elaboration of antitoxins. As suggested above, it is highly probable that the granular cells perform different functions in different species or even within any one species. Their wandering propensities suggest that they are capable of responding to diffuse stimuli operating in a fluid medium and occasioned by the several special needs of the organism. Their capacity to store glycogen in *Rutilus* and *Cyprinus* would partly meet the needs of fish whose food is rich in carbohydrates and may account for their absence in the carnivorous *Gobio* belonging to the same family, but this is obviously not the whole story and their activities in the numerous other fish in which they occur still remain to be discovered.

Discussion of Cytology

The free border of the intestinal cells in the lower chordates bears cilia which assist in the movement of food particles through the alimentary canal and is associated, in general, with ciliary feeding mechanisms. The evolution of the jaws produced a new mechanism for obtaining food and enabled the resulting gnathostomes to secure bulkier food; hence ciliated epithelium as a gut lining lost much of its original purpose and the result has been the evolution of a continuous free border pierced by fine canals as described by Baker for tetrapods and here for many teleosts. *Pterois volitans* and *Scarus sordidus* present free borders of unusual appearance, neither ciliated nor with typical canals, but show a condition that can be interpreted as intermediate between these extremes. It is highly desirable that an extensive survey of the free borders of the intestinal epithelium of lower vertebrates should be made to test this idea, and possibly to link the aberrant types together into an evolutionary scheme.

The fact that the free border is not of constant thickness throughout various parts of the intestine of the same fish suggests that this thickness can be, and normally is, altered from time to time—a feature probably concerned primarily with the absorption of digested food into the cells. Baker, indeed, goes farther and suggests that the opening and closing of the pores of the canals 'may be one of the reasons for the movements of the villi and an explanation of their musculature'.

The goblet cells produce both mucus and zymogen simultaneously and these products appear to pass together through the permanently open stoma into the lumen of the gut where they imbibe water, swell, and pass into solution. This permanent stomatal opening may well correspond to a specially enlarged canal of the free border of the absorptive cell and its narrowing and widening are probably influenced by movement of the intestinal folds in the same way.

Excellent comprehensive reviews on the roles of mitochondria and the Golgi element in animal tissues in general are given by such authors as Cowdry (1924), Bowen (1929), Bourne (1942), Hibbard (1945), and Worley (1946), and it is not proposed to extend that discussion beyond saying that the evidence obtained during the present investigation gives strong support to the view that mitochondria are intimately concerned with the process of fat absorption. The Golgi element also disintegrates during fat absorption (a finding supported by Cramer and Ludford, 1925, and by Liu, 1930), but this is certainly not the only role played by this structure. There is considerable agreement amongst previous workers, e.g. Brambell (1925), Bowen (1926, 1929), Ma (1928), and Worley (1944), that the Golgi element is concerned with secretion. Nassonow (1923) declares that spheres of mucus originate within it. Such evidence as the present investigation can supply is twofold: first the indirect evidence that the Golgi element is larger and better developed in the goblet cell than in the absorptive cell and secondly that the part of the cell where this structure lies may show a positive reaction to alkaline phosphatase (cf. p. 339).

The occurrence and peculiar staining reactions of the granular cells so characteristic of the gut of teleosts, and which, in spite of their diverse basiphilic and acidophilic properties, are probably generically comparable throughout, have already been discussed at length (pp. 331–6) and it is not proposed to re-enter that discussion here.

II. THE PHYSIOLOGY OF THE INTESTINAL EPITHELIUM

A. *Histochemical Studies*

1. *Hydrochloric Acid*

The presence of free acid was first demonstrated in the mammalian stomach by Claude Bernard and, although his procedure, involving the injection of potassium ferrocyanide into the animal, has been variously modified, all subsequent techniques employ the same basic principle (cf. Lison, 1936). The modification of FitzGerald (1910) is the one employed here. No precipitate of Prussian blue was found in the gut of any of the fishes examined and hence it may safely be inferred that free HCl did not occur. The fact that the injected ferrocyanide solution had indeed passed into the mucosa was confirmed when sections of the intestine were stained for glycogen by the Bauer-Feulgen technique, the chromic acid producing a copious blue precipitate in the epithelium and the tissue immediately subjacent to it. No other worker has so far succeeded in finding HCl in the cyprinid gut; thus one may safely conclude that the stomachless cyprinid has lost one of the principal attributes of the stomach and has not evolved acid digestion in any other organ.

2. *Lipase*

Gomori (1945) has recently succeeded in demonstrating the intracellular sites of lipase activity by histochemical methods. He used as a substrate either palmitic or stearic esters (known respectively as 'Tween 40' and 'Tween 60', manufactured by the Atlas Powder Co. of America). The substrate used in the present study was a gift from Dr. Gomori himself and is a 'product No. 81 of the Ornyx Oil & Chem. Co., Jersey City, U.S.A.'. Dr. Gomori, in his letter, described it as 'a stearic acid ester of a glycol of high molecular weight which is an excellent substrate for the histochemical demonstration of lipase'. For full details of the method reference should be made to Gomori's paper, but in essence it consists of first splitting the substrate by means of the lipase in the tissues, then precipitating the acid radicle as the calcium salt. This is then converted into the corresponding lead salt which, by treatment with ammonium sulphide, is finally transformed into the dark brown or black lead sulphide. Thus, on counterstaining with haematoxylin, dehydrating and clearing in dichloroethylene (*not xylene*) the sites of the original lipase activity may be clearly seen as shapeless blotches, dark brown or black in colour, of no constant size or shape which, under the oil-immersion, are seen to have a granular texture, the granules sometimes fusing into larger masses. Treatment of the slide before incubation with Lugol's iodine solution for one minute completely inhibits the reaction and thus provides a convenient method of control.

Positive results were obtained in all three cyprinids in the mucosal epithelium and in the pancreatic alveoli surrounding the intestine, while some lead sulphide was also deposited in the lumen of the intestine. The absorptive cells show the highest concentration of the enzyme (Pl. III, fig. 16), but a few goblet cells also show very meagre deposits of lead sulphide which in no way correspond to the zymogen granules. It is possible that the enzyme may have got there by diffusion rather than by secretion, especially since it also occurs free in the intestine. The important conclusion to be drawn from this result is that the absorptive cells are secretory as well as absorptive.

The highest concentration of the enzyme occurs in *Gobio*, where the mucosal folds are all loaded with the deposit. However, it decreases in intensity in a cranio-caudal direction. A similar general picture was obtained for *Cyprinus* and *Rutilus*, but the lipase activity in the third limb of the intestine and the rectum is very weak and it may even be absent entirely from these portions of the gut in *Rutilus*.

During the biochemical estimation of enzyme activity (p. 345) extracts from the wall of the buccopharynx were found to give a positive reaction for lipolytic enzymes. The histochemical test was accordingly repeated several times and on several individuals but was entirely negative except in one instance, namely, a piece of material from the pharynx of *Cyprinus*, deposits of lead sulphide occurring in the connective tissue amongst the adipose cells scattered in the wall. It is well known that fat-storing tissues contain lipase (Maclean, 1943), nevertheless Gomori (1946) failed to get positive results with his test on the adipose tissues of rhesus monkey, dog, and guinea-pig, although he succeeded with similar tissue from the rat and rabbit. Gomori also found that the stratified epithelium of the oesophagus of several species of mammals contains lipase; the stratified epithelium of cyprinids, however, proved entirely negative. The possible causes of this discrepancy between histochemical and biochemical results is further discussed on p. 345.

3. *Phosphatases*

It is to Gomori again that we owe satisfactory methods for the histochemical demonstration of both alkaline phosphatase (Gomori, 1939) and acid phosphatase (Gomori, 1941*b*). These methods have been used in the present work and the reader is referred to Gomori's original papers for technical details. Reference should also be made to Danielli's critical review (1946). All experiments were run with appropriate controls, namely, control of the method by running sections of rat kidney and fish kidney through the solutions simultaneously with those of the gut and control of any possible pre-existing calcium in the tissues by passing identical slides through the various stages but *omitting* the incubation stage. Any calcium present will react with the lead nitrate and produce a deposit, whereas phosphatase will not do so unless incubated with a substrate.

All tests for acid phosphatase were negative. Alkaline phosphatase was most abundant in the free border of the absorptive cells of all species (Pl. III, fig. 14). The superficial layer appears jet black, the canal and granular layers paler, the 'clear zone' still paler, while the rest of the cytoplasm showed no appreciable coloration. In *Rutilus* and *Gobio* the whole intestine from swelling to rectum exhibits this activity, although with considerably decreasing intensity in the more caudal regions. *Cyprinus* gives a somewhat similar picture except that the decrease in intensity is even more striking, the rectum showing hardly any phosphatase activity at all. Phosphatase activity is also seen in the subepithelial connective tissue; the fibres take a light brown colour while, in *Cyprinus*, the collagen fibres lying just internal to the circular muscle appear brownish-black. Blood corpuscles react positively and the granular cells of *Rutilus* and *Cyprinus* also show some activity (Pl. III, fig. 13). In *Gobio* the stratum compactum contains alkaline phosphatase (Pl. III, fig. 14).

The Golgi zone showed a positive reaction to the tests in a few absorptive cells only (Pl. III, fig. 15), so that it cannot be considered a constant feature in the cyprinid gut.

The walls of the bucco-pharynx contain considerable quantities of the enzyme. Its activity is greatest in the basal layer of the stratified epithelium and decreases gradually towards the surface (Pl. IV, fig. 17) (cf. also Al-Hussaini, 1948).

The results described above confirm the conclusions of Gomori (1941*a*), Kabat and Furth (1941), Bourne (1943), and others concerning the concentration of the enzyme in the free border of the absorptive cells, but they are at variance with the results of Emmel (1945) and Deane and Dempsey (1945) who consider that the location of the enzyme in the Golgi zone is a constant feature of intestinal epithelia.

4. *Absorption*

In order to demonstrate any possible differences in the absorptive capacity of different regions of the intestine or in the intestines of the different fish, it was felt that the absorption of fat would be likely to yield the most conclusive results as it is a process that has been much studied in mammals though little in fish. Herwerden (1908) during the course of his study of gastric digestion studied the absorption of fat from the stomach of several fishes, as did Greene (1913) in the king salmon and Dawes (1930) in the plaice.

The technique adopted here has been to fast the fish for some days and then administer, usually through the mouth, a small quantity of castor oil. The fasting period needs to be lengthy (2 weeks for *Gobio*, 3 for *Rutilus* and *Cyprinus*) since the fish intestine does not become empty so quickly as that of mammals. Food usually leaves the intestinal swelling fairly soon, but it takes much longer to clear the gut entirely (this agrees with other workers on stomachless fishes, cf. Barrington, 1942). It is important to change the water in which the fish live once or twice daily during the fasting period to prevent the fish from eating their own faeces.

According to Cameron (1945) castor oil is the glyceride of ricinoleic acid (a hydroxy derivative of oleic acid). It is not proposed to enter into a detailed discussion concerning the various implications and theories of fat absorption, but rather to give a straightforward account of the phenomenon as it could be seen in the three cyprinid fishes investigated.

Only two simple techniques were used to demonstrate fats, namely, (1) impregnation by means of osmium tetroxide by either Schridde's or Kolatchev's method (it is important to realize that OsO_4 has only a morphological, not a histochemical value, cf. Lison, 1936), (2) a modification of Ciaccio's method for 'lipoids' (= lipines, cf. Lison). The modification consists of staining by Sudan III in 90 parts 80 per cent. alcohol and 10 parts acetone instead of in 95 parts alcohol and 5 parts acetone. This was found to be more satisfactory for fish material. Although, according to Lison, Kaufman and Lehmann have criticized this method they accept it for non-saturated fatty acids; ricinoleic acid is such an acid and the method is therefore suitable for its detection.

With the osmium tetroxide techniques the absorptive cell in the fasting condition displays a dense granular cytoplasm (due mainly to mitochondria which are sometimes impregnated with OsO_4) and a Golgi element. A similar picture is obtained with Ciaccio's method except that the Golgi element is masked.

During active digestion of a normal diet the absorptive cells appear vacuolated when studied by methods other than those designed to preserve and stain fat (Pl. IV, fig. 18). Dawes (1929) found that the 'columnar cell' during the resting phase shows a deeply stained mass which becomes smaller during the active phase. He believes that the appearance of vacuoles is directly related to the presence of food in the lumen. Rogick (1931) thinks that the vacuoles represent a phase in the formation of goblet cells; McVay and Kaan (1940), however, exclude the possibility but offer no alternative explanation of the phenomenon. On the basis of experience gained during the present investigation a more probable explanation would seem to be that the 'vacuoles' simply represent the loci occupied by fat globules, the fat having been dissolved out by the alcohols and xylene used in preparing the sections.

It is convenient to describe the condition of the epithelium during fat absorption by means of seven protocols each describing a different experimental procedure and its result.

Protocol 1. A fasting roach 14·0 cm. long was given about 1 c.c. castor oil through the mouth and killed 19 hours afterwards. Transverse sections of various parts of the intestine were prepared and treated by Kolatchev's and Schridde's osmium techniques.

Fat globules of very variable size are found in the absorptive cells (Pl. IV, fig. 19); some may attain a diameter of 8 μ while others measure less than 1 μ. The free border is quite clear of globules and the clear zone is almost free from them. Next to the clear zone there are usually a few globules of small diameter followed immediately by globules of the largest size; indeed the large globules are confined to the luminal half of the cell. Here and there between the clear zone and the nucleus minute globules also are encountered, but the majority of them are found in the basal half of the cell.

The cells from the various parts of the intestine have not absorbed equal quantities of fat. In the intestinal swelling the greatest absorption has occurred on the crests of the mucosal folds, although the cells as far as halfway down the sides of the folds may contain fat. In the second limb of the intestine almost every cell is loaded with fat, while in the third limb, although almost every cell contains fat globules, their concentration is not so dense as in the second limb. In the rectal epithelium practically every cell is clear of fat.

The tunica propria also contains fat globules, but they are not as abundant as in the absorptive cells. The globules here are usually small, but sometimes shapeless masses, apparently formed from fused globules, may be seen in the meshes of the connective tissue. These masses are not usually so heavily impregnated by osmium as the globules themselves. Fat globules are extremely rare within the submucosa.

Protocol 2. A fasting roach 18·4 cm. long was given about 1 c.c. of castor oil by mouth and killed 43½ hours later. Transverse sections of various parts of the intestine were prepared and the fat was stained by Schridde's and Ciaccio's methods.

More cells were loaded with fat in the intestinal swelling than in protocol 1; even those at the bases of the mucosal folds contained fat. In general the fat globules were smaller and none was so large as 8 μ diameter. The second and third limbs present pictures comparable with those obtained before (Pl. IV, fig. 20). This time the rectum has also absorbed some fat, mostly along the crests of the folds. By comparison with the state of affairs in the fish killed only 19 hours after feeding it may be seen that many fat globules have passed inwards towards the nucleus (Pl. IV, cf. fig. 19 with fig. 20).

Small fat globules are still of frequent occurrence in the tunica propria but are not as numerous as formerly. In addition to the fat globules stained red, unstained 'vacuoles' also appear in the sections prepared by Ciaccio's method, but these 'vacuoles' do not appear in the material osmicated by Schridde's method, thereby suggesting that some of the fat at least is altered during the absorptive process.

Protocol 3. A fasting gudgeon 11·0 cm. long was given about 1 c.c. of castor oil by the mouth and killed about 21 hours afterwards. Transverse sections of various parts of the intestine were prepared and the fat stained by Kolatchev's and Ciaccio's methods. With the osmium impregnation (Kolatchev's method) a picture was obtained closely comparable with that described in protocol 1, except that some of the cells on the crests of the mucosal folds of the rectum have absorbed fat. Large masses of fat also occurred in the blood-vessels and lymph spaces in the tunica propria. With Ciaccio's method Sudan positive globules of varying sizes occur in the absorptive cells on the crests of the mucosal folds, being most abundant in the basal portions of the cells.

Protocol 4. A fasting gudgeon 11·4 cm. long was given about 1 c.c. of castor oil and killed 67 hours later. Transverse sections of the intestine were prepared as in protocol 3. Only a small number of fat globules were present. They were recognizable by either method and were present throughout the intestine but occupied only the crests of the mucosal folds.

Protocol 5. A fasting mirror carp 10 cm. long was given about 1 c.c. of castor oil by the mouth and killed 4 hours later. Transverse sections of the various parts of the intestine except the rectum were prepared by Kolatchev's technique. The absorptive cells showed considerable accumulations of fat, and, as in protocol 1, the larger globules were found in the luminal halves of the cells. Abundant fat globules were also present in the tunica propria.

Protocol 6. A fasting mirror carp 12·5 cm. long was given about 2 c.c. of castor oil through the anus and killed 21 hours later. Sections of the intestine were prepared as in protocol 3. In the osmicated preparations the resulting picture was similar to that in protocol 1, but by Ciaccio's method globules stained red by the Sudan III occurred in the intestinal cells but not in the connective tissue.

Protocol 7. A fasting mirror carp 15 cm. long was given about 0·2 c.c. of castor oil through the mouth and killed 21 hours later and sections of the gut were prepared as in the previous example. This time only the absorptive cells on the crests of the mucosal folds of the intestinal swelling showed any sign of fat globules.

The following conclusions may be drawn from the above experiments:

1. Fat is mainly absorbed by the second and third limbs of the intestine, the intestinal swelling absorbing less and the rectum very much less; indeed the amount absorbed by the rectum must be very small (protocols 1, 2, 3, 4, and 6). From protocol 7 it might appear that when the quantity of fat is very small it is entirely absorbed by the intestinal swelling, but much more supporting evidence would be needed before one could feel justified in making this generalization.
2. Early in the fat-absorbing process fat globules of widely varying size are found, some reaching considerable dimensions (8 μ diam.), while later they become more uniformly small (protocols 1, 2, 3, 4, 5, and 6).
3. The fat passes from the absorptive cells into the tunica propria, either into blood-vessels or lymph spaces. The submucosa contains little or no fat (protocols 1, 2, 3, and 5).
4. Some modification occurs to at least some of the fat during the absorptive process since the pictures obtained by means of the osmium techniques show a greater concentration of globules than those obtained by the more selective Sudan III methods (protocols 2 and 6).

5. *Glycogen*

For the demonstration of glycogen three methods were employed: (1) Best's alkaline carmine technique following alcohol-formol fixation. (For a discussion on the reliability of this method see Davies and Francis, 1941.) (2) Bauer-Feulgen's method, also after alcohol-formol fixation. (3) The method originally designed by McManus (1947) as a mucus stain but, as Dr. J. F. A. McManus has said in a personal communication to the author, the method seems to show carbohydrates and their compounds such as glycogen, mucins, &c. In each case the saliva test was used to differentiate between glycogen and galactogen, the former being hydrolysed thereby while the latter is not (Lison, 1936).

Glycogen was found only in the granular cells of *Rutilus* and *Cyprinus*, *Gobio* giving only negative results (cf. also Table 1).

B. *Biochemical Studies*

A study of the literature reveals that the estimation of enzymes in the intestine of the carp has yielded somewhat conflicting results. This seems to be due to the fact that the investigators have failed to realize, either wholly or partially, that the gall-bladder and intestine are intimately invested by fatty tissue containing pancreatic alveoli, and hence special precautions are needed to ensure that preparations of intestinal enzymes are not contaminated from this source. Thus, for example, Babkin and Bowie (1928) found a proteinase, a lipase, and an amylase in the bile of *Fundulus heteroclitus*, while Mackay (1929*b*) failed to find any enzymes at all in the bile of three different fishes when she took the precaution of ligaturing the bile-duct and immersing the gall-bladder in Bouin's fluid for a few minutes before withdrawing the bile. Bayliss (1935) achieved similar results with the plaice, but he used Zenker's fluid for 5 seconds to destroy the enzymes in the pancreatic alveoli.

Further, most workers have not defined clearly what they mean by 'anterior', 'middle', or 'posterior' intestine, and the use of these terms has hitherto been vague and without morphological foundation. The convenient divisions of the intestine into four regions has already been clearly defined in Part I of this work (Al-Hussaini, 1949, p. 334); they are the intestinal swelling or first limb, the second limb, the third limb, and the rectum. Each of these portions has been separately tested for enzymes and, in addition, samples of bile and the secretion of the mucous membrane of the mouth and pharynx have been included.

The procedure adopted was to free the gut from the mesenteries and surrounding tissues as completely as possible. The intestine was then removed from the fish, the bile-duct ligatured, and the intestinal contents washed out by a stream of saline. Ligatures were then applied delimiting the segments above referred to and the whole intestine was immersed in Bouin's fluid for 1 minute (half a minute in the case of *Gobio*) and then removed and washed rapidly in three or four changes of lukewarm saline and finally washed thoroughly in a current of running water for about 5 minutes, then dried on filter-paper. The segments were then cut apart, slit open longitudinally, and the mucous membrane scraped carefully away. The scrapings of each segment were then ground up separately with quartz sand and about their own volume of glycerin. In some cases the extract was allowed to stand for 24 hours at room temperature and in others it was used immediately. There was no significant difference in the results by either method. Owing to the smallness of the fish available (the carp weighed between 12 and 40 gr. and the gudgeon between 8 and 10 gr.) material from several specimens was pooled for each experiment, but even then the extracts from the rectum were so small as to render an assay impracticable. Again owing to the small quantity of material available filtering was avoided and the extract, after dilution with an equal volume of N/20 NaOH, was centrifuged and the clear supernatant fluid pipetted off into a tube. Aqueous extracts were also prepared.

Vonk (1927) found 38° C. to be the optimum temperature for the gut enzymes of the common carp, but Bayliss (1935) could find no difference in their behaviour in the plaice whether they operated at room temperature, 25° C., or 37° C. The present tests were all carried out at $37 \pm 1°$ C.

Vonk (1927) found the optimum pH for the gut enzymes of the common carp to be round about the neutral point, and it can be seen from Table 2 that both the secretion and the contents of the intestine are practically neutral

TABLE 2. *Acidity of Contents of Intestine, Bile, and Mucous Membrane of Buccal and Pharyngeal Cavities, expressed in terms of pH*

	Segment of gut						
Fish	*Bucco-pharynx*	*Intestinal swelling*	*Second limb*	*Third limb*	*Rectum*	*Bile*	*State of gut*
Rutilus	7·16	7·67	6·65	6·51	6·20	7·42	Plenty of food in intestinal swelling.
,,	6·86	6·80	7·25	6·52	7·00	6·85	Plenty of food in entire intestine.
Gobio	6·93	7·54	7·59	7·17	6·12	7·19	,,
,,	7·13	7·47	7·27	7·56	6·83	7·00	Intestine empty.
,,	6·56	7·59	6·46	6·90	6·30	7·20	Food in whole intestine except second limb.
,,	6·91	7·72	7·41	7·54	7·21	7·40	Intestine empty.
Cyprinus	6·25	6·27	6·56	6·44	6·68	6·17	,,
,,	6·69	7·70	6·73	7·55	6·67	6·72	Food in third limb and rectum.

in all three species. The addition of an equal volume of N/20 NaOH to the extract brought the pH to round about 7·5 and produced a definite pink colour with phenol red as an indicator.

The amylolytic activity was assayed by incubating the extract with an equal volume of 1 per cent. starch suspension, the activity being estimated visually by the method of Haslewood and Strookman (1939) (originally devised for estimating glucose in 0·05 ml. of blood) by which comparisons are made against the colour given by incubating an equal volume of starch solution with human saliva.

The proteolytic activity was estimated by the method of Cole (1933) which consists of incubating equal volumes of the extract with 1 per cent. casein solution and estimating the quantity of unaltered protein which remains after a definite time interval by precipitating it with 1 per cent. acetic acid.

The lipolytic activity was tested by the method of Anrep *et al.* (1925). This consists of incubating the enzyme extract with 1 per cent. glycerol-triacetate as substrate buffered to pH 7·8 with a phosphate buffer, the fatty acids liberated being estimated by titration against N/50 NaOH. This is a very sensitive method and gives quick results. Olive-oil emulsion was also tried as a substrate but was abandoned in favour of the more sensitive glycerol-triacetate.

In all tests a few drops of toluene were added and the incubation was carried on for 24 hours in a thermostatically controlled water-bath. Each experiment was controlled by testing against a tube containing the same substances, but which had been boiled to kill the enzyme. Extracts prepared without the preliminary precaution of fixing the pancreatic tissue and of the intestine and pancreas together were also tested for comparison.

TABLE 3. *Enzymatic Activity of the Alimentary Canal and Bile of* R. rutilus, G. gobio, *and* Cyprinus carpio, *symbolically expressed*

Part examined	*Enzyme*	*G. gobio*	*R. rutilus*	*Cyprinus carpio*
Buccal and pharyngeal walls	A	−	+	+
	P	−	−	−
	L	+	++	++
Intestinal swelling	A	+++	+++	++++
	P	++	++	+
	L	+++	+++	+++
Second limb of intestine	A	+	+++	++
	P	++	++	++
	L	++	++	++
Third limb of intestine	A	+	+	+
	P	++++	+++	+++
	L	−	Very slight	Slight
Bile	A	−	Very slight	Slight
	P	−	−	−
	L	−	−	−

A = Amylase. P = Proteinase. L = Lipase.

The results, given in Table 3, are expressed by symbols rather than numerically since it was felt that numerical results based on such small quantitites (from 1·0 to 2·0 c.c. of extract) of inevitably impure material might well result in the appearance of tempting comparisons and relationships altogether unjustified by the circumstances. The results obtained from the preparations in which no preliminary fixation of pancreatic tissue was done showed definitely stronger reactions, while from those in which the extract was made from intestine and pancreas together gave stronger reactions still.

From the results we may reach the following conclusions:

1. The results of biochemical tests for proteinases correspond with those of the histochemical tests for zymogen granules.

2. The biochemical estimation of lipase activity also corresponds pretty closely with the histochemical tests, with the exception of tissue from the bucco-pharynx, which gave positive results with the biochemical method but failed to do so (with the exception of one individual) with the histochemical one. Presumably this is due to qualitative rather than quantitative causes since the third limb of the intestine contains relatively very much smaller quantities of the enzyme, but this was easily detectable by both methods. It is of interest to note in this connexion that Maclean (1943) got biochemical evidence of strong lipase activity in the mammalian spleen, but Gomori (1946) failed to demonstrate it by his histochemical test. Vonk (1937) calls attention to the fact that the specificity of this group of ferments (lipases) is not very

pronounced, while Frazer (1946) says that 'lipase may be one enzyme . . . or it may consist of a group of esterases'. Further comparative work as between biochemical and histochemical results should yield data of considerable interest.

3. Both lipolytic and amylolytic enzymes show a maximum concentration in the intestinal swelling and decrease gradually towards the caudal end of the gut.

4. Proteolytic enzymes, on the other hand, are more concentrated in the third limb of the intestine than in the more cranial segments. Assuming the histochemical test to be a safe guide, the rectum is at least equal to the third limb in this respect (biochemical assay could not be applied to the rectum, *vide supra*).

5. Bile, apart from a very slight amylolytic activity in the herbivorous *Cyprinus* and omnivorous *Rutilus*, is practically devoid of enzymes.

6. The mucous membrane of the bucco-pharynx shows amylolytic activity in *Rutilus* and *Cyprinus* which, though slight, is stronger than that of bile. In all three forms it shows lipolytic activity. This is probably concerned with fat storage and with the utilization of fat stored in the submucosa rather than with digestive processes (cf. also pp. 338 and 339).

7. The concentration of carbohydrases is in direct relation to the relative amounts of carbohydrates in the food of the fish, being highest in *Cyprinus* and least in *Gobio*, while the protease activity is highest in *Gobio* and lowest in *Cyprinus*, being thus directly correlated with the amount of animal food the fish eats; this agrees with comparable results obtained by earlier workers. For example, both Kenyon (1925) and Vonk (1927) correlated the strength of the enzymes with the nature of the diet; Kenyon found that the hepatopancreas and the intestine of the common carp both secrete a much more powerful amylase than do those of the carnivorous pickerel, while Vonk found the strength of the proteinases in the common carp to be six to eight times weaker than those of the pike. Further, Ishida (1936), who investigated four different species of stomachless fish, found amygdalase and salicinase in herbivorous fish only, never in carnivorous. Finally, Grossman *et al.* (1944) have shown that by substituting glucose for corn starch in a balanced diet fed to rats an increase in the amylase content of the pancreas results.

Discussion of Physiology

Kenyon (1925), Vonk (1927), and Yonge (1931) have shown that digestion in fishes is essentially the same as in other vertebrates, but Babkin and Bowie (1928) distinguish between the acid-alkaline type in which a stomach is present and free HCl is accordingly secreted, and the exclusively alkaline digestion of the stomachless fishes. With the loss of a stomach, pepsin digestion is also lost, although Krukenberg (1877-8, quoted from Biedermann, 1911) claimed to have found that the mucous membrane of '*Cyprinus tinca*', in January, secretes a strong pepsin. Digestion in the three cyprinids which form the basis of the present study is exclusively alkaline.

The localization of enzymes in cyprinids has been the subject of much controversy, due mainly to inaccurate definition of the intestinal segments. Thus Luckhau (1878) found that the 'anterior intestine' secretes both amylase and proteinase and Knauthe (1898) demonstrated a strong proteinase in this part of the gut. Yung (1915) found that the entire intestine produces a weak proteinase as also does the hepato-pancreas. He claimed further that if these two weak enzymes are mixed together their action is considerably strengthened. Kenyon (1925) maintains that the intestinal secretions of the carp show only very weak tryptic digestion, and he thinks that, in such fishes with a diffuse pancreas, the trace of tryptic digestion found may be due to small ramifications of pancreatic tissue embedded in the intestinal wall. He identified the intestinal proteinase as consisting mainly of erepsin—as is normal in other vertebrates. Vonk (1937) believes that the intestinal mucosa of the carp can furnish an erepsin as well as an enterokinase, but that the amylase present comes from the diffuse pancreas. Finally Carrié (1937) asserts that the intestine of the tench produces no enzymic secretion at all, a finding unsupported by any other workers on this or related species.

Although no attempt has been made in the present study to identify individual enzymes in the three principal categories, yet it has been clearly shown that not only does the intestinal mucosa secrete enzymes, but that the various regions of the intestine exhibit this property in different degrees. It is suggested that these conclusions merit full consideration for two reasons, namely, the preliminary fixation of the pancreatic tissue precludes the possibility of the mucosal scrapings being contaminated from this source, and secondly the *in vitro* proteolytic and lipolytic biochemical tests are in accord with the histochemical investigation. Unfortunately no comparable histochemical technique for carbohydrates is available; if such could be devised it would fill the gap and make complete confirmation possible.

According to Biedermann (1911), Krukenberg (1878) has shown that the buccal mucosa of the carp secretes a diastase. Neither Knauthe (1898) nor Beauvalet (1933*b*) was able to confirm this. As can be seen from Table 2, the results of the present work lend support to Krukenberg's contention, since a diastase, though weak, is undoubtedly present in the mucosa of the bucco-pharynx of the mirror carp and roach, though it is absent from the gudgeon.

How far then has the loss of a stomach affected the balance of secretion? We learn from Beauvalet (1933*b*) that, according to his own results (1933*a*) and those of others, when fishes have a stomach the secretions of their intestines do not affect proteins to any appreciable degree, whereas in stomachless fish, this class of foodstuffs is capable of being digested along the entire length of the gut, a conclusion completely in accord with results of the present work. This finding is an important one, for it appears that the loss of a stomach and consequently of peptic digestion has been compensated by the development of a strong tryptic (? ereptic) enzyme by the intestine which augments strong enzymes of a similar character elaborated by the hepato-pancreas. Other cases of enzymic substitution are known to occur in fish, e.g. Beauvalet (1933*a*) compared the digestive capacity of the hepato-pancreas of *Ameiurus* and *Micropterus* and found it much higher in the former fish since the enzymes from the hepato-pancreas are augmented in *Micropterus* by those of the pyloric caeca, which structures are absent in *Ameiurus*. Chesley (1934) made comparable studies on other fishes and came to similar conclusions.

Since the stomach is also a receptacle for storing food it follows that a fish with a stomach can take in a greater mass of food at any one time than can a stomachless fish, making it necessary for the latter to feed at shorter intervals. This permits the digestive enzymes to mix more thoroughly with the food and consequently their action is accelerated (cf. Barrington, 1942). Thus the elaboration of tryptic enzymes by the intestine and more frequent feeding resulting in their more efficient use offers a dual compensation for the loss of the stomach.

The function of alkaline phosphatase has attracted a host of workers during the last decade. Evidence that phosphatase may be directly related to glucose absorption was first advanced by Lundsgaard (1933). Indirect evidence that this enzyme is concerned with absorption is offered by the present investigation which has shown that alkaline phosphatase is especially concentrated along the free border of the absorptive cells, that is, in direct contact with the digested food. Its presence in the tunica propria may well be associated with the passage of food materials from the absorptive cells into the body fluids. According to Bourne (1943) it is not known for certain whether the phosphorylation of sugars is carried out in the lumen of the intestine or whether the sugar is first adsorbed on to the surface of the cell, or absorbed into the brush border (canal layer), phosphorylated, and then passed into the interior of the cell.

The relation between alkaline phosphatase and glycogen is also a generally accepted thesis. Loci of glycogen in various tissues have been shown to be also sites of phosphatase activity, as, for example, in the liver (Gomori, 1941*a*), placenta (Wislocki and Dempsey, 1946), in the hair (Johnson and Bevelander, 1946). In the present work a similar association between phosphatase and glycogen has been observed in the granular cells of *Rutilus* and *Cyprinus*.

The stratified epithelium of the buccopharynx also contains alkaline phosphatase, the highest concentration being in the stratum germinativum. It might be argued, on the grounds of the above hypothesis, that the enzyme might here act in an analogous way to that in the free border of the absorptive cell or the brush border of the kidney tubule, but this time by absorbing glucose from the tunica propria rather than from the lumen. In such epithelia as that lining the buccopharynx the intercellular substance is very scanty, and the intake of glucose for purposes of nutrition may perhaps be effected thus by a chemical process of phosphorylation rather than by the physical process of diffusion (cf. Al-Hussaini, 1948).

For the tremendous literature on fat absorption the reader is referred to Verzár and McDougall (1936), Frazer (1946), and Nielsen (1946). Frazer reviews two hyoptheses of fat absorption: (1) the lipolytic hypothesis advanced by Verzár and McDougall and (2) the partition theory put forward by Frazer himself. About the first he says 'it maintains that fat is completely hydrolysed in the intestinal lumen, that the intestinal cell has an outer pavement membrane, that paraffins are not absorbed, etc.'. Frazer's own hypothesis is based on the partial hydrolysis only but the complete emulsification of fat. Thus he believes that fat globules of less than 0·5 μ in diameter may be absorbed without being hydrolysed, and that the intestinal absorptive cell has a porous free border (a point amply confirmed by the present work) which would allow the passage of such minute globules through its canals so that paraffins, which are not affected by lipase, can be absorbed. As regards the phosphorylation of fat, the lipolytic theory claims that it is a stage in the resynthesis of fat, while according to the partition hypothesis it is not concerned with resynthesis but occurs at the oil/water interface, producing an essential change in the interfacial structure, the phospholipid being added to the fat particles in the intestinal wall.

So far as the present investigation is concerned one of the main results is the demonstration that fat is first dealt with by the cells on the crests of the mucosal folds and that absorption later spreads down the sides of the folds to their bases. There does not appear to be a direct relationship between the amount of fat absorbed and lipase content (as demonstrated histochemically) of the tissue absorbing it. Thus the lipase activity, as shown by both histochemical and biochemical assay, is highest in the intestinal swelling and lowest in the third limb and rectum, but experimental evidence suggests that the greatest amount of fat is absorbed by the second limb. Evidence has been brought forward to suggest that lipases may vary qualitatively and that lipase may be detectable biochemically, and thus be physiologically significant, yet fail to yield positive results with the histochemical test employed.

The fact that no fat globules have been encountered in the free border might appear to discount the partition theory, since this is essentially based on the passage of fat globules through the canals of the canal layer; on the other hand, it may be that this passage is accomplished very rapidly and hence evades histological demonstration.

A number of observations have been made concerning fat globules in the tunica propria. Dawes (1930) found Sudan positive globules in this tissue in the plaice while Herwerden (1908), working with various elasmobranchs and teleosts, has described the transfer of fat globules from the epithelial cells of the stomach into the lymph spaces accompanying blood-vessels. Barton (1902) working on kelts, and Greene (1913) on the king salmon, found that fat absorbed by the intestinal mucosa passed into the tunica propria. The results of the present work are entirely in agreement with these findings and have shown that fat is transferred from the absorptive cells of the intestine to the lymph and blood-vessels of the tunica propria to be further transported to the fat depots.

ACKNOWLEDGEMENTS

In addition to the acknowledgements made in Part I of this work which I should like to repeat here with renewed gratitude, I have to thank Professor D. H. Smyth and Dr. Q. H. Gibson of the Physiology Department and Mr. R. E. Davies of the Department of Biochemistry for their help in the biochemical part of the work. My thanks are also due to Dr. G. Gomori of Chicago University for sending me a sample of 'Product No. 81' of the Ornyx Oil & Chemical Co., New Jersey, and to Mr. J. Noel Wood, General Manager of the Sheffield Corporation Water Works, for supplying the fish used in this work. For the photomicrographs I am again indebted to Mr. J. Hancock of this Department.

I express my gratitude to Dr. E. T. B. Francis and to Prof. L. E. S. Eastham for much valuable help in arranging my material for publication.

SUMMARY

1. The free border of the intestinal absorptive cells of cyprinids is nearly similar to that in tetrapods. This border has been described for four other species of fish and the possible evolution of the border from a ciliated epithelium has been described.

2. The mitochondria of the absorptive cells are arranged in a supranuclear group of mainly rod-like bodies and a subnuclear group of mainly granular ones.

3. The mitochondria are coarser and the Golgi element more readily impregnated with osmium tetroxide in the carnivorous *Gobio* than in the omnivorous *Rutilus* or the herbivorous *Cyprinus*. In all three fish both organoids are associated with fat absorption.

4. The goblet cells secrete both mucus and zymogen. Their stomata are permanently open.

5. Granular cells are described in *Rutilus* and *Cyprinus*, and in seven other species of fish, but they are absent from *Gobio*. Their varying reactions to numerous cytological techniques are described.

6. Alkaline phosphatase is especially concentrated in the free border of the absorptive cells. It occurs along the whole length of the gut. Its possible role in the absorption of fat and glucose is discussed.

7. Lipase is secreted by the absorptive cells. Fat absorption occurs along the whole length of the intestine, but the second limb of the intestine is especially active in this respect.

8. Carbohydrases and lipases are more concentrated towards the anterior end of the gut, whereas proteinases are more abundant caudally.

9. Carbohydrases are richer in *Cyprinus* and *Rutilus* than in *Gobio*.

10. Zymogen and lipase secretions are more concentrated in *Gobio* (carnivorous) than in the other two species.

11. The pH of the intestine of all three fishes is round about neutral point. Free HCl is absent from all three.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. A composite drawing of the intestinal epithelium of *G. gobio* giving a synthetic picture of the cytoarchitecture as revealed by various techniques.

Fig. 2. Vertical section of the intestinal epithelium of *R. rutilus* showing the canal layer of the free border. Ciaccio's technique. ×2,100.

Fig. 3. Tangential section of the free border of *R. rutilus* showing the intercellular bands. Fixative: M.A.S. of Baker; stain: Heidenhain's haematoxylin-eosin. ×2,100.

Fig. 4. *R. rutilus*; two adjacent folds in contact with one another and their free borders greatly expanded. Note canals, intercellular bands and supranuclear group of mitochondria. Champy-Kull method. ×1,500.

Fig. 5. Vertical section of the intestinal epithelium of *R. rutilus* showing intercellular bands. Fixative: M.A.S. of Baker; stain: Masson's trichrome. × 1,500.

Fig. 6. Vertical section of the intestinal epithelium of *Pterois volitans*, showing the free border. Fixative: Zenker-formol; stain: Masson's trichrome. × 1,500.

Fig. 7. Vertical section of the duodenal epithelium of *Scarus sordidus* showing the free border. Fixative: Regaud's; stain: Bensley–Cowdry. × 1,500.

PLATE II

Fig. 8. Vertical section of the intestinal epithelium of *G. gobio* showing the Golgi element (G.E.) and nucleus (N.) of the absorptive cells. Kolatchev's method. × 2,100.

Fig. 9. Vertical section of the intestinal epithelium of *G. gobio* showing a goblet cell (G.C.) and its Golgi element (G.E.) and nucleus (N.). Kolatchev's method. × 2,100.

Fig. 10. *Atherina forskali*, section of submucosa, showing the granular cells. Fixative: Bouin; stain: haematoxylin-eosin. × 1,200.

Fig. 11. *R. rutilus* showing the granular cells. Fixative: Bouin; stain: haematoxylin-eosin. × 1,200.

PLATE III

Fig. 12. *R. rutilus*, section of submucosa, showing granular cells. Fixative: alcohol-formol; stained for glycogen by McManus's method. × 1,200.

Fig. 13. *R. rutilus*, section of submucosa showing granular cells containing alkaline phosphatase. Gomori's method. × 850.

Fig. 14. Transverse section of intestinal wall of *G. gobio* showing location of alkaline phosphatase. Gomori's method. × 85.

Fig. 15. Vertical section of a few absorptive cells of *R. rutilus* showing location of alkaline phosphatase. Gomori's method. × 1,200. Note phosphatase located in Golgi zone, indicated by arrow.

Fig. 16. Transverse section of a part of a fold of the intestine of *G. gobio* showing location of lipase. Gomori's method counterstained lightly with haematoxylin and eosin. × 620.

PLATE IV

Fig. 17. Vertical section of the mucous membrane of the pharynx of *G. gobio* showing location of alkaline phosphatase. Gomori's method. × 620.

Fig. 18. Vertical section of a few absorptive cells from the intestinal mucosa of *R. rutilus* during active digestion. Fixative: alcohol-formol; stain: haematoxylin-mucicarmine. × 1,200. Note the presence of a vacuole (V) in the luminal half of each cell.

Fig. 19. Vertical section of a few absorptive cells from the mucosa of the second limb of the intestine of *R. rutilus*, showing fat globules. Schridde's method. × 1,000 (see Protocol 1, p. 341).

Fig. 20. Transverse section of the apex of an intestinal fold of the intestine of *R. rutilus* showing fat globules. Schridde's method. × 1,000 (see Protocol 2, p. 341).

ABBREVIATIONS USED IN PLATES

C.L.	canal layer.
CL.Z.	clear zone of cytoplasm.
G.C.	goblet cell.
G.C.M.	goblet cell showing mucus.
G.C.Z.	goblet cell showing zymogen.
G.E.	Golgi element.
GR.L.	granular layer.
I.B.	intercellular band.
L.M.	lymphocyte.
N.	nucleus.
P.C.G.	'pear-shaped' cell showing Golgi element.
P.C.R.	'pear-shaped' cell showing rods.
S.C.	stratum compactum.
SB.M.	subnuclear group of mitochondria.
SF.L.	superficial layer.
SG.L.	subgranular layer.
SP.M.	supranuclear group of mitochondria.
V.	vacuole.

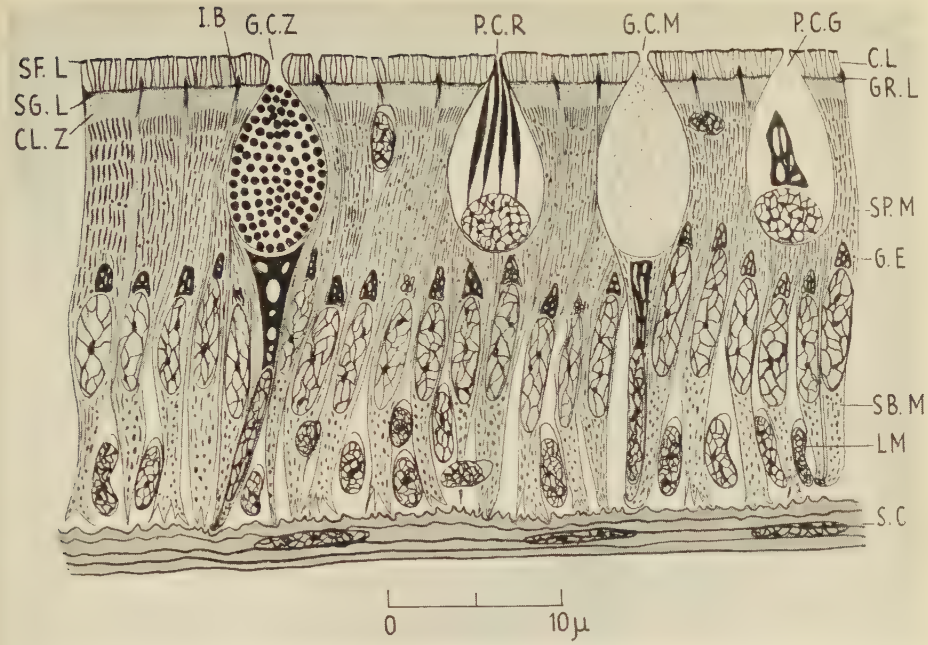
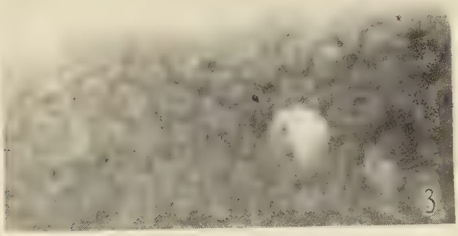
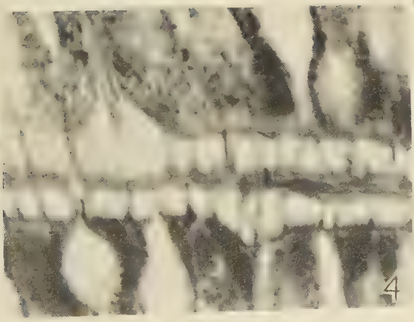
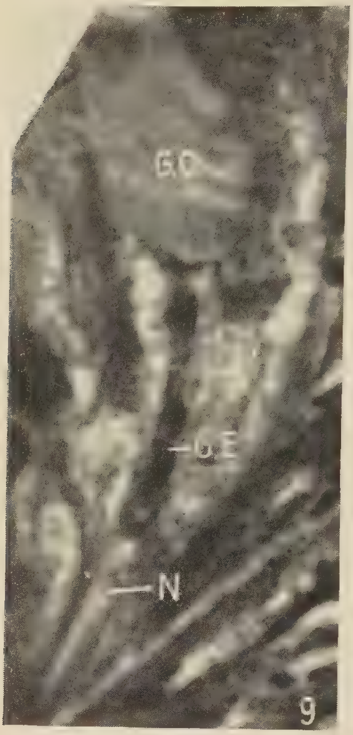


FIG. 1





AL-HUSSAINI—PLATE I



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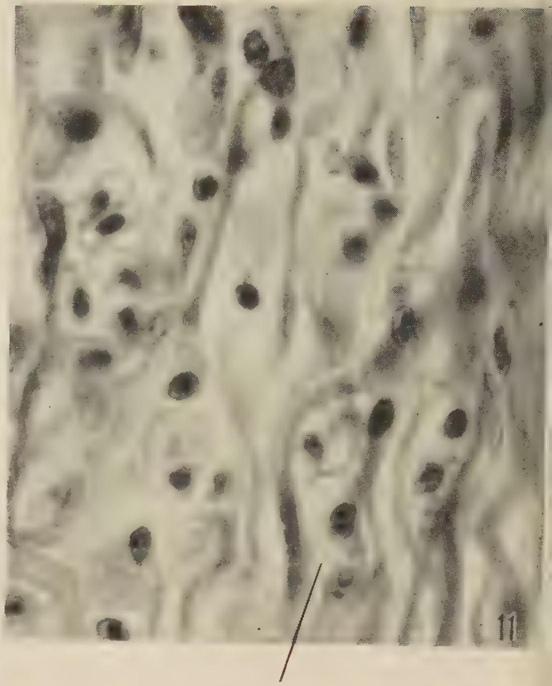

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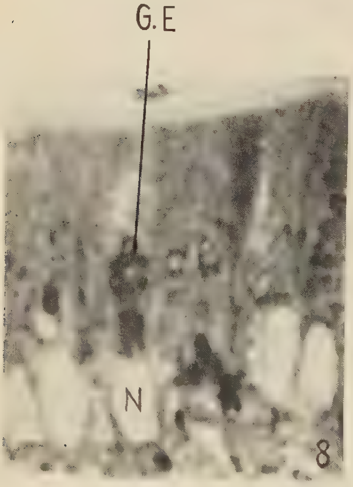

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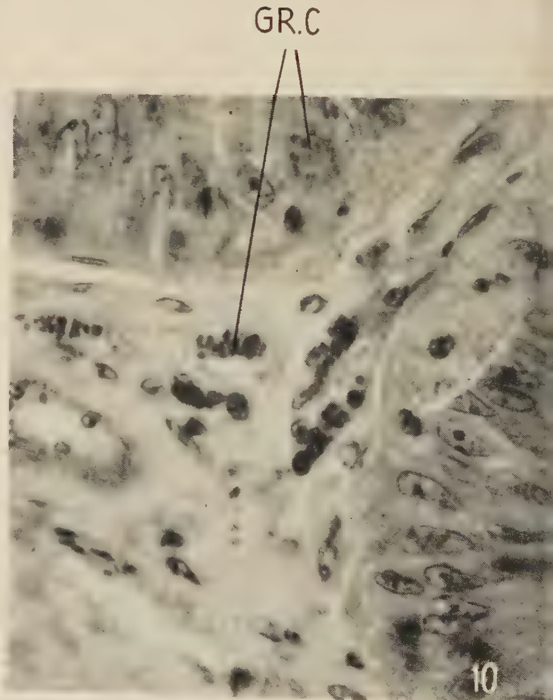

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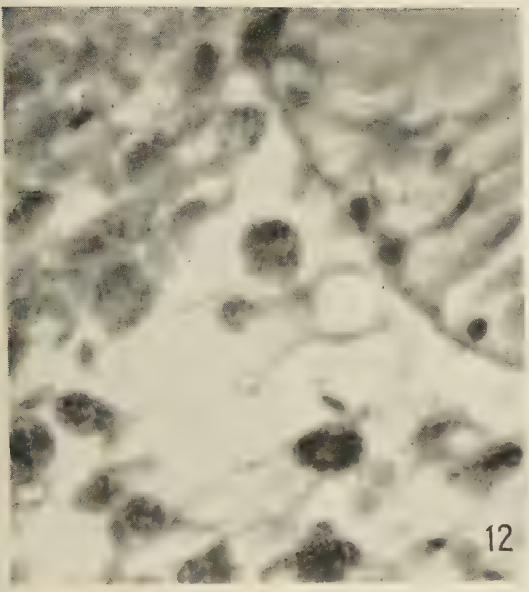
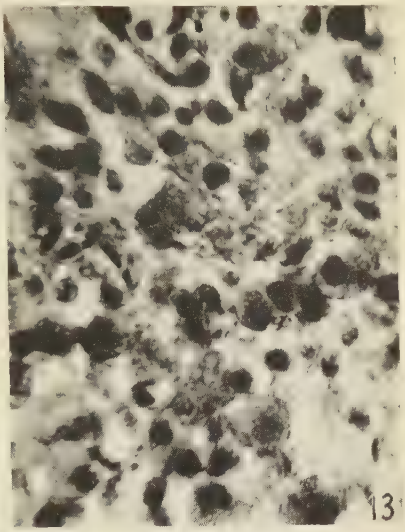
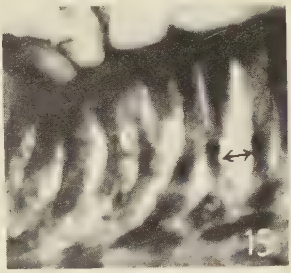
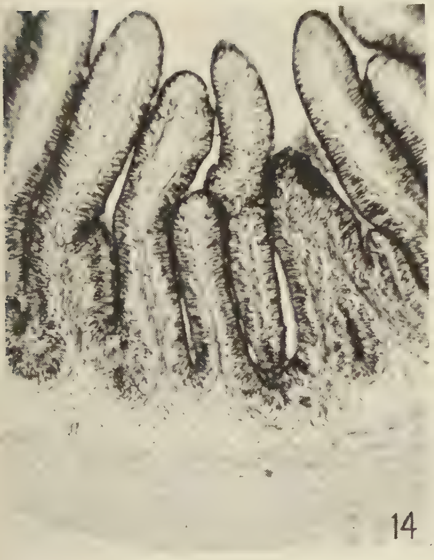
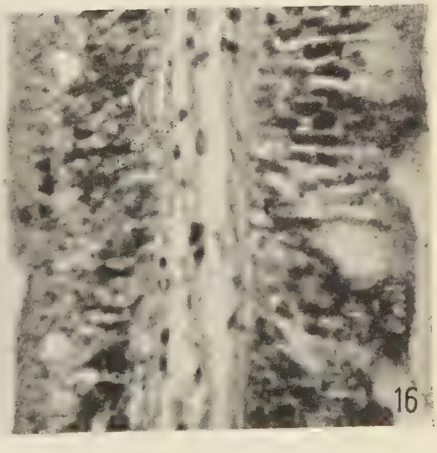
AL-HUSSAINI—PLATE II



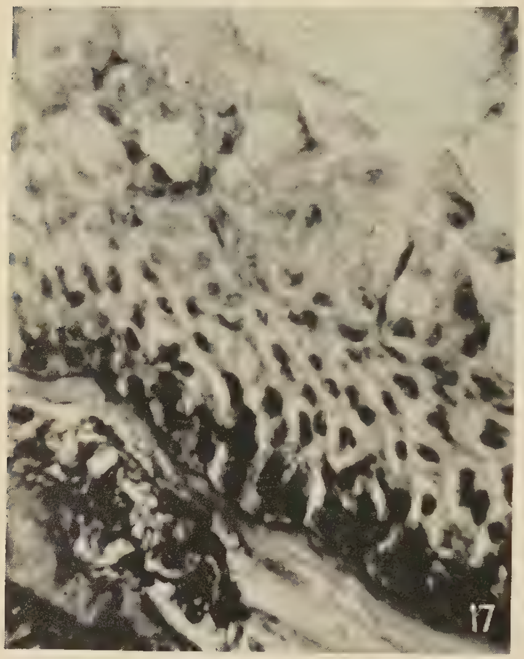


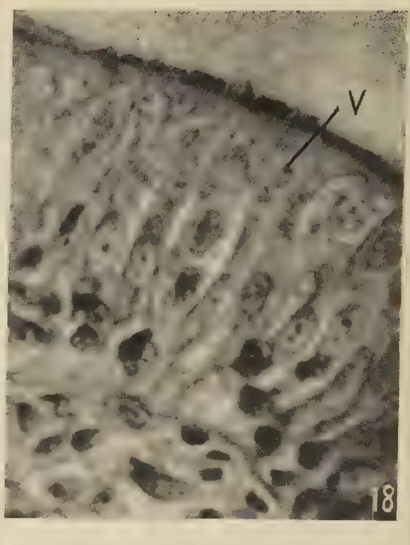


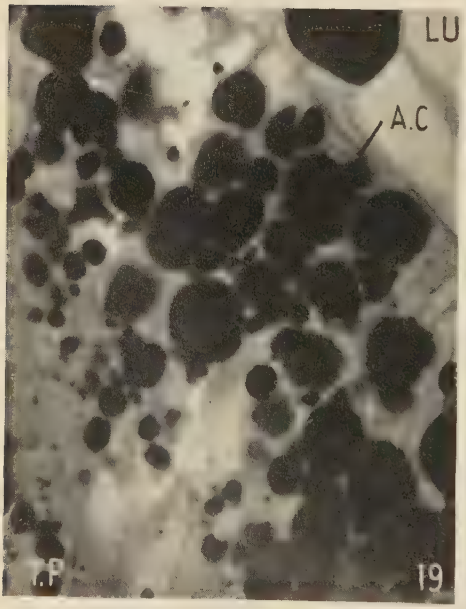


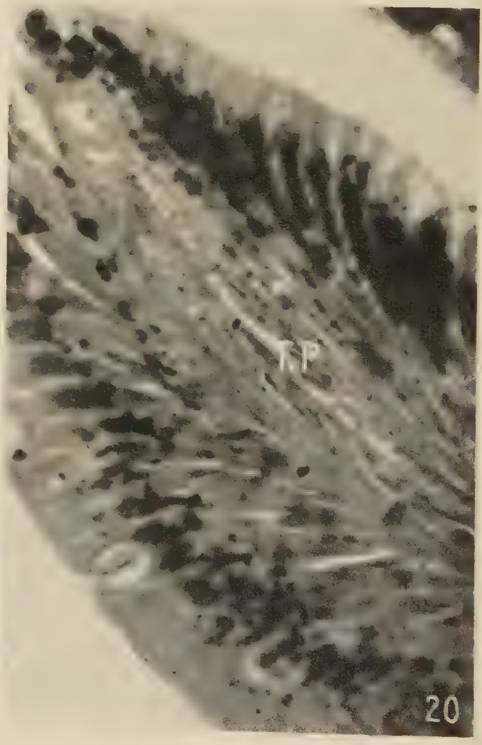


AL-HUSSAINI—PLATE III









AL-HUSSAINI—PLATE IV

Mitosis in the Mouse:
A Study of Living and Fixed Cells in Tissue Cultures

BY

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AND

A. F. HUGHES

(Sir Halley Stewart Fellow)

(From the Strangeways Research Laboratory, Cambridge)

With eight Plates

Introduction

By means of phase contrast ciné-photomicrography applied to tissue cultures it has become possible to study the division of living vertebrate cells in much greater detail than hitherto. Observations have already been made by these methods on mitosis in chick cells (Hughes and Swann, 1948; Hughes and Fell, 1949) and in amphibian tissue (Hughes and Preston, 1949). The present authors have extended these investigations to mammalian cells, using cultures of new-born mouse tissues, mainly of the spleen and kidney. In this material prophase can be identified in the living cultures at a very early stage and mitosis followed to its completion. Mouse cultures are characterized by a wide variation in cell size; investigations showed that the larger cells are polyploid. The first part of this paper describes the course of normal mitosis and is based on the study of both living and fixed preparations; the second part records the evidence for the polyploid nature of the large nuclei.

Normal mitotic cell division in tissue cultures has been investigated by several authors. One of the earliest accounts is that of Strangeways (1924) who made warm-stage observations on mitosis in chick fibroblasts. The earlier work on mitosis in tissue culture has been fully reviewed by Levi (1934). Some more recent studies by W. H. Lewis (1940) are discussed in the text.

The literature on the chromosome cytology of the mouse has been cited by Grüneberg (1943); the most recent publication on this subject is that of Matthey (1936). In *Mus musculus* the diploid number of chromosomes is 40 and the attachment to the spindle (centromere) is subterminal.

In their research H. B. F. was responsible for the tissue culture and the preparation and study of the fixed material. The photography was by A. F. H. and the analysis of the film records was the joint work of both authors.

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December, 1949.]

MATERIAL AND METHODS

Tissue Culture

Tissue fragments from the spleen, kidney, and heart of mice killed 3 to 21 days after birth were explanted in large hanging drop cultures on No. 2, $1\frac{1}{4}$-in. square coverslips which were sealed with molten paraffin-wax to $3 \times 1\frac{1}{2}$-in. hollow-ground slides. The culture medium consisted of one drop of fowl plasma mixed with one drop of 10 to 12-day chick embryo extract; in the later experiments the plasma was diluted with an equal volume of Tyrode before use, so as to provide a softer clot and thus facilitate the emigration of the cells. The cultures were transferred to fresh medium at 3- to 4-day intervals.

After several subcultures the tissue was transplanted to a No. 1 coverslip and embedded in a plasma embryo extract clot in the usual way. A square hole was then cut in the clot, one side of the square coinciding with the edge of the tissue; fluid from the clot immediately exuded into the space. The coverslip was mounted on the special metal chamber described by Hughes and Swann (1948) and the preparation was placed in the incubator. When the tissue began to grow, the outgrowth extended into the clot on three sides, but on the fourth side it advanced over the glass in a thin, expanded sheet of cells covered by the fluid medium in the square hole. Mitosis was active in this thin layer of cells which were excellent for optical study. For phase-contrast photography this simple device was a great improvement both on cultivation in a complete clot in which the emigrating cells are in many different planes and where there is optical interference from the fibrin network, and on cultivation in an entirely fluid medium which makes the preparation awkward to handle and in which the least disturbance or change in temperature may cause a complete or partial retraction of the outgrowth.

Fixation and Staining

The cultures were fixed for a few minutes either in Maximow's fluid[1] or in 2 per cent. osmium tetroxide, usually after being rinsed in warm Tyrode; it is important carefully to drain off the surplus saline in order to ensure instantaneous coagulation of the cells. Tissue cultures are ideal material for cytological purposes, because their extreme thinness allows of almost perfect fixation (Pl. I, fig. 2). The cultures were washed for some hours in several changes of distilled water and were stored for not more than a few days in 80 per cent. alcohol.

Nearly all the cultures were stained by Feulgen's method, after which the central explant was removed leaving only the zone of outgrowth; this was done to reduce the total thickness of the final preparation and so ensure critical illumination during microscopical study. The cultures were counterstained in a mixture of 0·1 per cent. light green+0·1 per cent. naphthol green in 90 per cent. alcohol, and after being dehydrated and cleared in xylene, were mounted whole in Canada balsam or Gurr's DePeX mountant on very thin slides. A few preparations were made in which the cultures were hydrolysed as for Feulgen's method and then stained in well-ripened Ehrlich's haematoxylin (Hughes and Fell, 1949).

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[1] 10 parts Zenker's solution; 1 part formol; 1 part 2 per cent. osmium tetroxide.

Photography

The photographic records of the cultures were made at two magnifications. Fields of up to 100 cells were photographed at a total magnification of × 50. These low-power pictures were taken on 35-mm. film with apparatus designed and used by the late Dr. R. G. Canti and loaned by the British Empire Cancer Campaign. The Canti machine is now installed at the Strangeways Laboratory and has been modified for phase-contrast photography. The records were made on Kodak 'Microfile' film with a × 10 phase-contrast objective; the source of light was a ribbon filament lamp, with a green filter. The interval between exposures was either 1 or 2½ minutes; photography was continued for 2 days.

Single cells were photographed at high magnification on 16-mm. film by a technique which has already been described in detail (Hughes, 1949). Usually a cell in early prophase was chosen and was followed through mitosis for 1–4 hours. The interval between photographs varied from 1 to 6 seconds.

All the objectives and condensers used were manufactured by Messrs. Cooke, Troughton & Simms, Ltd.

Analysis of Film Records

The analysis of the film records is one of the most important and difficult parts of the cinematographic technique. Usually projection at 16 frames per second is too rapid for the detailed study of particular features in cellular processes. On the other hand, the observation of individual frames one at a time is inadequate, as projection in motion is necessary to appreciate the interrelation of adjacent structures.

Recently a 'Craig' Projecto-Editor, generously presented to the Laboratory by the Rockefeller Foundation, has proved of the utmost value in film analysis. With this instrument motion projection at any speed in either direction can be combined with the viewing of single frames. In our opinion this is a necessary piece of apparatus to obtain the best results from film records.

Comparison of the Living with the Fixed Nucleus

In some cultures the same cell was photographed in three different ways: (1) by phase contrast in life, (2) by phase contrast after fixation in 2 per cent. osmium tetroxide, and (3) by ordinary illumination after being stained by either Feulgen's method or Ehrlich's haematoxylin. The procedure was repeated with cells at different stages of mitosis. Two sets of such photographs are shown in Pl. I.

Fixation with osmium produces an almost negligible amount of distortion of the nucleus as is shown by the photographs of an interphase nucleus on Pl. I, fig. 2. The chromocentres and chromonemata (see p. 359 below) are in focus in both fig. 2*a* and fig. 2*b*, Pl. I, and occupy identical positions in both photographs. After fixation the Feulgen-positive chromocentres are no longer visible by phase contrast, but of course reappear when the cell is stained. The same behaviour is shown by the chromosomes during mitosis. In Pl. I, fig. 1*a*, a spleen cell was photographed at the first moment of anaphase; by the time it had been fixed the daughter groups of chromosomes had separated to the extent shown in Pl. I, fig. 1*c*. In Pl. I, fig. 1*b*, taken by phase contrast after fixation, the chromosomes are invisible and the whole spindle area is lighter than the surrounding cytoplasm, densely packed with mitochondria. The radial arrangement of the latter at the poles of the spindle is shown in both fig. 1*a* and fig. 1*b*, Pl. I.

INTERPHASE

General Appearance of the Cells

Most of the observations were made on the reticular cells of spleen cultures. When fragments of the spleen are first explanted large numbers of lymphocytes and monocytes emerge; these are followed more slowly by the reticular cells which predominate over other cell types after several passages. They are very suitable for phase-contrast photography as they spread out thinly on the glass and divide actively. The fibroblasts in heart and kidney cultures were also studied but less extensively.

Spleen reticular cells and kidney fibroblasts are of very similar appearance in cultures; the cells of both types vary considerably in size. When the cells are growing in a fluid medium on the surface of the glass (see p. 356), the membranous border of the cytoplasm is often actively motile when seen in a speeded-up film, and may show pinocytosis (Lewis, 1931), i.e. the ingestion of globules of the external fluid which pass into the cell as a stream of clear vacuoles.

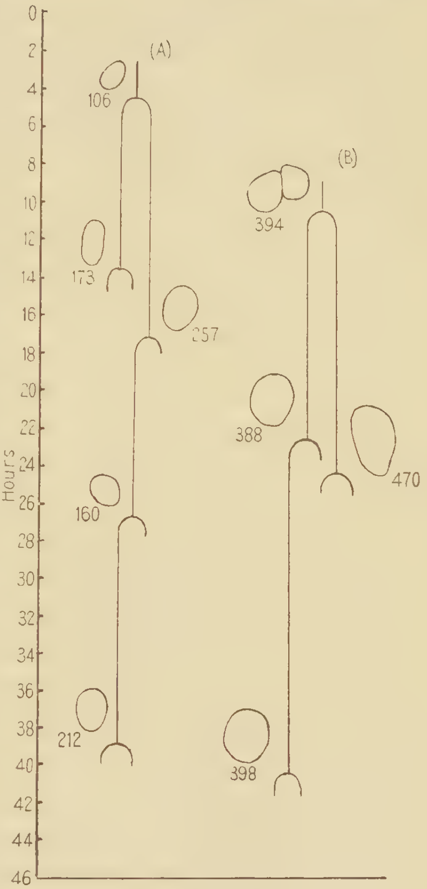
The marginal cytoplasm is often quite clear except for the filamentous mitochondria which are exquisitely seen under the phase microscope. Nearer the nucleus fatty globules and, especially in the spleen cells, particles of ingested matter are aggregated round the cytocentre. If too abundant these granular inclusions may obscure the details of mitosis, particularly in the large polyploid and binucleate cells (see below), as they cause a glare in the phase microscope due to phase reversal.

The cytocentre is sometimes hidden by the large granules, but it is often visible in life as a grey, finely granular area to one side of the nucleus. In some cells it has a clearly defined, rounded outline as in a binucleate cell figured by Hughes in a previous paper (Hughes, 1949; Pl. II, fig. 3).

Nuclear Cytology

In both living and fixed cultures, the nuclear structure of spleen reticular cells or kidney fibroblasts is beautifully clear in those cells which are expanded flat on the coverglass. The interphase nucleus, which is usually oval or rounded, contains from 3 to 7 nucleoli varying greatly in size and shape. In life the nucleoli appear dark grey by phase contrast and do not change their form and relative positions; sometimes they contain vacuoles similar to those described by Lewis (1943).

Feulgen-positive heterochromatic granules, the chromocentres, which look almost black in the living cell, are stuck to the nucleoli and to the inner surface of the nuclear membrane; a similar appearance has been described by Ludford, Smiles, and Welch (1948) in mouse tumour cells. These chromocentres range in number from about 10 to 72; when only a few are present they are much larger than when there are many, which suggests that the large bodies are formed by the fusion of several small heterochromatic granules. In Table I are listed the number of chromocentres in 21 interphase nuclei. Five nuclei (marked) were much bigger than the rest; one of them had only 25 chromocentres which, however, were very large, but the remaining 4 contained many (36–72). In Feulgen preparations of well-fixed cells the ground substance of the nucleus, which stains pale pink, seems almost homogeneous at first sight. Close inspection with a high-power apochromat, however, shows it to consist of a mass of faintly granular filaments, the chromonemata, which are particularly clear in cultures fixed with 2 per cent. osmium tetroxide (Pl. II, fig. 4). The chromonemata are attached to the chromocentres and are always more distinct near the point of attachment than elsewhere; parts of the chromonemata are visible in life in a large, well-flattened cell (cf. Ludford *et al*, 1948), especially near the union of the threads with the chromocentres. There appears to be considerable variation in the visibility of chromocentres and chromonemata in interphase nuclei. When searching in living cultures for cells in early prophase we have often found nuclei containing obvious filaments and granules and have followed them for



TEXT-FIG. 1. Diagram of cell lineages from a spleen culture, photographed at low power. Each ⅄ represents a mitosis, and the vertical lines intermitotic periods. For each parent nucleus in early prophase the outline is given, together with the nuclear area in square microns. (*a*) Lineage of a cell with a medium-sized nucleus. (*b*) Lineage of a binucleate cell, the descendants of which had large nuclei.

as long as 1 hour, during which time they remained unchanged. Such nuclei may possibly be approaching mitosis.

TABLE 1. *Table showing the Variation in the Number of Chromocentres in Different Nuclei*

K = kidney culture. Those unmarked are spleen cultures.
L.M. = large mononucleate cell.

Interphase	*Prophase*	*Daughter nucleus*
10	30	20
14	32	22
16	38	22
16	39	22
K 17	40	K 23
18	40	24
K 19	K 44	27
21	44	31
23	48	35 L.M.
K 25	54	K 54 L.M.
25 L.M.	..	..
26	..	..
27	..	..
28	..	..
28	..	..
29	..	..
34	..	..
K 36 L.M.	..	..
K 42 L.M.	..	..
62 L.M.	..	..
72 L.M.	..	..

The Intermitotic Period

From 6 low-power records of the growth of mouse spleen cultures one film which covered a period of 50 hours was selected for intensive study. When photography began there were about 20 cells in the field, and when the record ended there were about 70. Fifty-one mitoses were counted which were remarkably evenly spaced throughout the 50 hours.

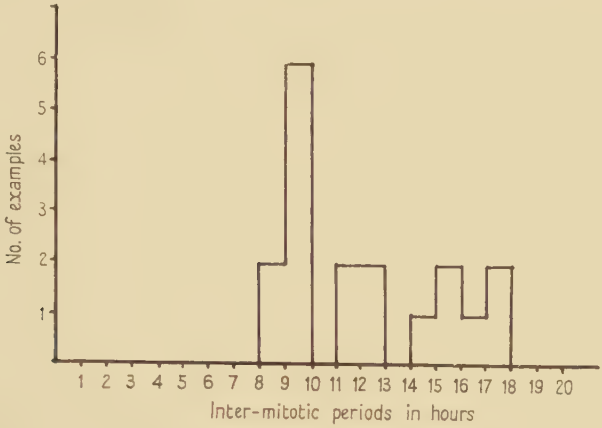
Whenever possible the daughter cells produced by each division were traced forward through the film until they entered their next division or could no longer be identified. Eighteen intermitotic periods were thus recorded, and one cell was followed through four generations (Text-fig. 1). The intermitotic intervals ranged from 6 to 18 hours with a mode of 9 hours (Text-fig. 2). There was no correlation between the size of a nucleus at prophase and the time which elapsed before its daughter cells divided.

MITOSIS

I. *Diploid Mitosis*

The onset of *prophase* is characterized by an increase in the number and a diminution in the size of the heterochromatic granules (Pl. II, figs. 5 and 6;

Table 1). The chromonemata seen in the interphase nucleus become much more distinct and are now clearly visible in the living cell, though at this stage they are less sharply defined with phase contrast than are the mitochondria (Pl. V, fig. 31*a*). As the nucleic acid charge on the chromosomes increases the heterochromatic granules can no longer be counted accurately, as they cannot always be distinguished with certainty from bends and kinks in the long threads. The bends and kinks diminish, however, as the threads contract and the picture becomes much less confused.



TEXT-FIG. 2. Histogram showing distribution of intermitotic periods. Data from the same spleen culture as in Text-fig. 1.

Some of the chromosomes radiate from the nucleoli (cf. Lewis, 1940), but many are applied to the inner surface of the nuclear membrane where their structural details can be studied. One end of each thread is occupied by one of the heterochromatic granules which stains more intensely with Feulgen's method than the rest of the chromosome (Pl. II, fig. 7) and behind which the thread is often seen to be split; in a living chromosome the terminal heterochromatic granule is well shown. The chromosomes often appear finely banded both in the living nucleus and in Feulgen preparations (Pl. II, fig. 8); possibly the bands represent gyres in the contracting thread.

Whether all the chromosomes terminate in a heterochromatic granule is uncertain, as it is only occasionally possible to find a free end unobscured by neighbouring threads. It can be stated, however, that whenever a heterochromatic region can be clearly observed it is found to occupy the end of a chromosome, and that when both ends of the same chromosome can be distinguished only one has a heterochromatic granule. It is also uncertain whether the heterochromatic region is near the centromere, which is subterminal in the mouse, or whether it forms the distal end of the chromosome.

A direct morphological continuity between the mitotic chromosomes and the chromocentres of the interphase nucleus was observed in living cells in tissue culture by W. H. Lewis (1940). This author, however, does not distinguish between the Feulgen-positive chromocentres and the Feulgen-negative nucleoli and applies the latter term to all intranuclear granules. It seems clear, however, that the 'small nucleoli' of Lewis which were seen to be in structural continuity with certain chromosomes correspond to the chromocentres in our material, and that the chromosomes which seemed to develop from the large nucleoli in his cells were, in fact, associated with the chromocentres sticking to the surface of the nucleoli.

Our films show that in early prophase the nucleus often revolves. The rotation usually occurs in greatly expanded cells and seems to be associated with the spindle, since the asters often appear shortly after it has taken place. It is possible that the nucleus is rotated by the spindle material forcing its way between the nuclear and cell membranes, which in very flattened cells are separated by a mere film of cytoplasm. As the chromosomes acquire their nucleic acid charge the heterochromatic region ceases to be distinguishable, and the longitudinal split dividing each chromosome into two chromatids is now obvious in stained cultures (Pl. II, fig. 8). In the living cell the split in the chromosomes is usually first seen nearer metaphase (Pl. V, fig. 31*c*).

In favourable cells the formation of the asters can be followed in some detail (Pl. V, fig. 31*b*); the process is particularly well seen in polyploid cells (Pl. VI, fig. 32*c*). They first appear at the poles of the early prophase nucleus as rounded areas of dark granular cytoplasm similar to that of the cytocentre of interphase. In these areas the mitochondria and other cell inclusions soon acquire a radial orientation. The spindle is formed from the asters and sometimes each half of the developing spindle produces a deep indentation of the nuclear membrane (Pl. VI, fig. 32*c*). A few seconds later the nuclear membrane suddenly collapses and disappears, the nucleoli finally vanish and the cell enters *metaphase* (Pl. V, fig. 31*c*; Pl. II, figs. 10 and 11). At the time when the nuclear membrane vanishes the whole cell contracts. The extended processes are withdrawn, and the surface area is reduced. In the living cell the spindle is seen as a clear area surrounded by dark, rather granular cytoplasm (Pl. V, fig. 31*d*). The chromatids of each pair are now clearly separated except at the centromere (Pl. II, fig. 10), and for several minutes they move at random up and down the spindle (cf. Lewis, 1939; Hughes and Swann, 1948). In mouse cells the radial stage, characteristic of early metaphase in chick fibroblast cultures (Hughes and Fell, 1949), is only occasionally seen (Pl. II, fig. 9). In the chick cells the spindle probably forms at right angles to the plane of the cover-slip and then rotates through 90° until it is parallel with the cover-slip, whereas in the mouse cells the spindle usually develops in the plane of the cover-glass from the beginning and there is no subsequent rotation. When a radial arrangement of the chromosomes is seen in exceptional mouse cells the asters are not visible, being above and below the metaphase plate.

The chromosomes soon become orientated near the equator, and after an interval of 6 to 20 minutes *anaphase* begins (Pl. V, fig. 31*f*; Pl. II, fig. 12): the chromatids separate and pass to opposite poles of the spindle (Pl. V, fig. 31*g*, *h*). A few minutes later the cytoplasm begins to constrict at the equator (Pl. V, fig. 31*h*, *i*) and the cell enters *telophase*. Both anaphase and telophase are accompanied by the usual cytoplasmic 'bubbling'.

Close attention was paid to the process of *nuclear reconstruction* as it is shown in Feulgen preparations, in the films, and by direct observation of the living cells. The development of a living daughter nucleus from telophase until the end of the first few hours of interphase is illustrated in Pl. VII, fig. 34; the area of this nucleus (see p. 367) is plotted against time in Text-fig. 3.

As soon as the daughter chromosomes reach the poles they shorten and become closely packed together (Pl. V, fig. 31*h*, and Pl. II, fig. 13). Suddenly this chromosomal mass begins to swell; at the same time it unravels into a web of fine threads radiating in all directions from the oblong areas of refractile, chromatinic material which represent the original chromosome bodies (Pl. VII, fig. 34*c*) and which in fixed preparations are still strongly Feulgen-positive (Pl. II, figs. 14, 15).

In the film records it is sometimes possible to follow for a short time the transformation of one or two individual daughter chromosomes in the compact group. The chromosome becomes very short and plump, then vacuoles appear in its substance and it rapidly dilates (cf. Lewis, 1940); at the same time a delicate lattice-work of threads, closely associated with that of neighbouring chromosomes, is drawn out of the shrinking chromatinic material. At the interface between the chromosome and the cytoplasm, a sector of the nuclear membrane develops.

In Feulgen preparations the vacuoles which form in the interior of the chromosomes make them appear double (Pl. II, fig. 15), but we are doubtful whether this apparent doubleness does, in fact, represent a split in preparation for the next mitosis. The problem is being investigated further.

At first the young nucleus is a narrow body with an irregular outline, but as it continues to swell it assumes a smooth, oval contour (Pl. VII, fig. 34*c*). The chromatinic areas become less refractile in the living cell and in fixed preparations are seen to have lost much of their Feulgen positive charge, except in the terminal heterochromatic segment (Pl. IV, fig. 25). They are rapidly replaced, however, by a more diffuse system of irregular lumps and strands which occupy the greater part of the nucleus and which are much more clearly seen in the living nucleus (Pl. VII, fig. 34*e*, *f*) by phase contrast than in Feulgen preparations. The subsequent history of this system shows that it represents the nucleolar material.

When the film records are traced backwards, the nucleolar material seems to form at the sites of the original chromosomes, but the close study of an unusually favourable record indicated that the chromatic material disappears before the nucleolar substance becomes visible. The surface of the developing nucleoli is studded with dark heterochromatic granules from each of which fine filaments emerge, so that the nucleolar components are knit together by an intricate mesh of threads (Pl. VII, fig. 34*d*, *g*). Other heterochromatic granules adhere to the nuclear membrane. It is noteworthy that throughout the nucleus most of the threads appear taut, as if under tension. The heterochromatic granules are intensely Feulgen-positive in fixed preparations; although most of the chromosomes have now shed nearly all their nucleic acid charge, a few of those attached to the nucleoli lost it much more slowly than the others.

The larger nucleolar masses form where the greatest number of chromosomes are aggregated. As time goes on, the nucleoli become even more refractile and less diffuse (Pl. VII, fig. 34*g*, *h*).

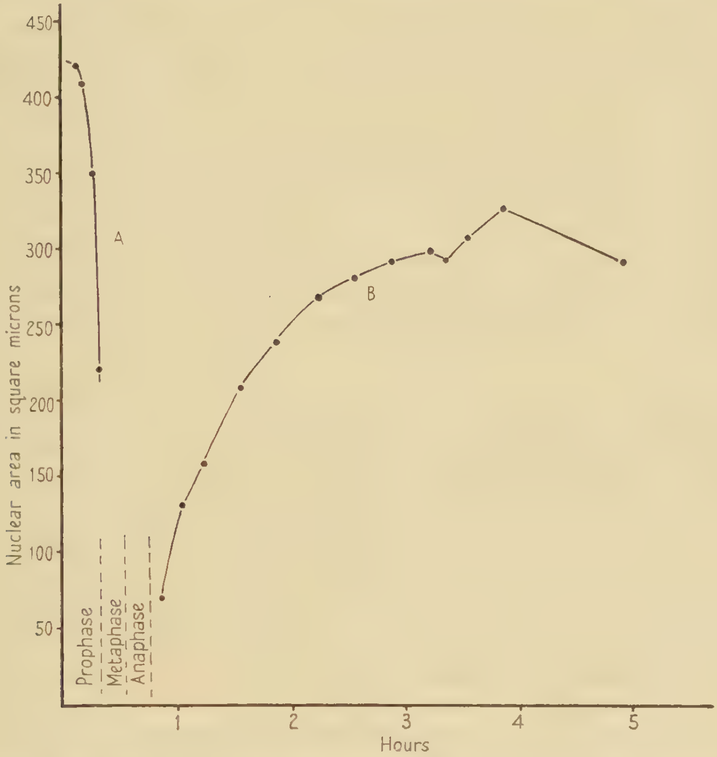
Although the nucleus continues to swell, during the later stages of reconstruction there is little, if any, increase in size of the nucleoli, which therefore occupy a diminishing proportion of the nuclear volume. As a result, there is an extensive rearrangement of the nuclear contents (Pl. VII, fig. 34*i*–*k*) which is interesting to watch in the film records. Sometimes two nucleolar masses which were originally close together move apart and the chromosome threads connecting them are then drawn out to several times their previous length; elsewhere the nucleolar substance may retract from the margin of the nucleus, leaving behind a chromocentre stuck to the nuclear membrane. Meanwhile the nucleoli change their appearance; as they become more refractile, the diffuse, straggling nucleolar system of the young daughter nucleus becomes transformed into the compact, rather rounded masses characteristic of interphase (Pl. VII, fig. 34*k*). At the same time the chromosome threads become less conspicuous and the chromocentres to which they are attached more distinct both in life and in Feulgen preparations (Pl. IV, fig. 27). Finally the nucleus ceases to enlarge and the mature interphase condition has been reached (Text-fig. 3).

We tentatively suggest the following interpretation of some of the appearances seen in nuclear reconstruction. In early telophase the compactly arranged chromosomes swell, presumably by imbibition of water, and are thus brought into even more intimate contact than before. The nuclear membrane forms at the interface between the chromosomes and the cytoplasm; the nucleus then rapidly dilates and the chromosomes are forced apart by the expansion. At the same time the chromosomes begin to lose their nucleic acid charge and their structure becomes looser, as indicated by the small vacuoles which appear within them (cf. Wilson, 1925, pp. 134–8). The chromosomes seem to adhere to each other, to the nuclear membrane, and to the developing nucleoli, so that when they are thrust apart by the dilation of the nucleus, it is possible that their complex adhesions may cause loops of their chromonemata to be dragged out in all directions by the intranuclear tensions and thus form the delicate web of threads so beautifully seen in the living as well as in the fixed cell (Pl. IV, fig. 25; Pl. VII, fig. 34*d*); as mentioned above, the general straightness of the threads suggests that they are being held taut by mechanical forces. It must be emphasized that this explanation of the arrangement of the fine filaments is mere hypothesis as the fibres are too near the limits of resolution for their detailed structural relations to be determined by ordinary microscopical observation.

II. *Heteroploid Mitosis*

The spleen reticular cells and kidney fibroblasts in the zone of outgrowth may be classified in three groups:

1. Mononucleate cells of medium size which are probably diploid and which form the greater part of the cell population (Pl. II, figs. 3 and 4).



TEXT-FIG. 3. Nuclear area of a cell during mitosis, (*a*) that of the parent cell in prophase, (*b*) that of one daughter cell in telophase and early interphase (this daughter cell is shown in Pl. VII).

2. Large mononucleate cells which appear to have more than the normal number of chromosomes, i.e. to be heteroploid (Pl. III, figs. 16, 18, and 19).
3. Binucleate cells with either medium or large nuclei (Pl. III, fig. 17).

The observations we are about to describe suggest that these groups are not distinct cell types but are produced by variations in the mitotic process.

1. *The formation of a binucleate cell.* Of 51 mitoses recorded in a spleen culture photographed at low magnification for 50 hours, one produced a binucleate cell (Pl. IV, fig. 30, compare Pl. IV, figs. 24 and 29). Division was apparently normal, but cytoplasmic cleavage was not completed and the daughter cells reunited (cf. Strangeways, 1924; Fell and Andrews, 1927).

If the incidence of mitosis without effective cleavage is of the order of 1 in 50, as this record would suggest, the rate of formation would not account for all the binucleate cells seen in the outgrowth during a single 3- to 4-day passage. It is probable that many binucleate cells survived from previous passages and some may have emigrated from the explant.

In normal mitosis the daughter cells often remain connected by the interzonal strand for some time (Pl. V, fig. 31*j*) and while such continuity persists, reunion is always possible. Binucleate cells can be artificially produced by exposing a dividing cell to strong illumination which often suppresses cleavage completely.

2. *Mitosis in binucleate cells.* Six records of binucleate cells in prophase have been obtained, in all of which one spindle was formed and both sets of chromosomes joined a single metaphase plate (Pl. VIII, figs. 36 and 37). One of the cells was fixed at this stage. In two records anaphase was followed by cleavage and two abnormally large mononucleate daughter cells resulted (Pl. VIII, fig. 36); one of the films was made under low magnification and the daughter cells were followed through two subsequent generations of mononucleate cells (see p. 360 and Text-fig. 1).

In three of the records, mitosis did not end in cleavage and a binucleate cell was again formed. The chromosomes were very distinct in one of these cells which contained few cytoplasmic granules (Pl. VIII, fig. 37). They became arranged on a metaphase plate where they remained for 35 minutes; an imperfect anaphase ensued in which the chromatids moved an abnormally short distance apart and in which one half of the spindle seemed to be defective, since both groups shifted in the same direction across the field. During the next half-hour daughter nuclei were reconstructed from the tetraploid groups of chromosomes. In this way a cell with two diploid nuclei was converted into one with two tetraploid nuclei.

In a fixed preparation a cell was encountered containing two separate spindles each with a metaphase plate, but unfortunately we have no ciné records of mitosis of this type. There may be other unrecorded variants of mitosis in binucleate cells.

We have no evidence of the fusion of nuclei in binucleate cells during interphase. Very large nuclei containing many nucleoli have occasionally been seen in which the nuclear membrane showed an equatorial infolding which might have represented a stage in the fusion of two nuclei; although one such nucleus was watched for several hours it gave no indication that fusion was in progress.

3. *Mitosis in large mononucleate cells.* Several records were made of the division of large mononucleate cells similar to those which may result from the fusion of a binucleate element. The appearance of the nuclei at prophase suggests that they contain more than the diploid number of chromosomes (Pl. VI, fig. 32*a* and *b*).

In living cells, several details at this stage were more clearly seen in heteroploid than in diploid elements. Thanks to the larger size of the former, it was easily possible to focus separately on the interior of the nucleus and at the level of the nuclear membrane (Pl. VI, figs. 33*b* and *c*). The position of chromonemata and chromocentres on the inner surface of the nuclear membrane was thus quite clear. The asters appeared either in polar indentations of the nuclear membrane as described above (Pl. VI, fig. 32*c*), or asymmetrically to one side of the nucleus (Pl. VIII, fig. 35*c*).

Between prophase and metaphase the movements of the chromosomes in relation to the asters and spindle were clearly seen in these large nuclei (Pl. VI, fig. 32 *c–f*). Before the nuclear membrane had vanished the chromosomes crowded towards the two poles, giving a spurious effect of anaphase (Pl. VIII, fig. 35*b*; Pl. III, fig. 21). When the membrane had gone and the spindle had formed, the numerous and rather widely scattered chromosomes moved towards the spindle (Pl. VI, figs. 32*c–d*). These facts suggest that the chromosomes are drawn in some way towards the asters and spindle. The spindle was of normal size, and during metaphase seemed over-crowded with chromosomes. Anaphase, telophase, and cleavage proceeded normally in such cells, and two large daughter cells were produced in the example shown in Pl. VIII, fig. 35.

Attempts were made to count the chromosomes in fixed and stained preparations of these large cells. Tissue cultures are very unfavourable for chromosome counts, however, because as already stated the metaphase plate usually lies at right angles to the plane of the coverslip. All our counts were almost certainly too low, but they showed that many more than the diploid number of chromosomes were present.

4. *Observations on nuclear size.* The results described above implied that in cells of the same histological type, the size of the nucleus, at the same point in the mitotic cycle, is related to the number of its chromosomes (compare Pl. II, fig. 10, and Pl. IV, fig. 22; also in Pl. IV, fig. 25 with fig. 26, and fig. 27 with fig. 28).

The areas of a number of living nuclei in early prophase, including some of the largest, were measured in the following way. From each of twenty-two records of mitotic cells made at high magnification, a frame in prophase was selected before the nuclear membrane had begun to contract. This was projected at a total magnification of ×2,000, and the area bounded by the nuclear membrane was measured with a planimeter. The nuclear volume could not be estimated as it is impracticable to measure the depth of so thin a structure as the nucleus of a flattened tissue culture cell. It is reasonable to assume, however, that a relationship exists between the volume and the area of the projected nuclear image, which for convenience is termed 'the nuclear area'. The measurements were restricted to well-flattened spleen reticular cells.

The results are listed in Table 2. It will be seen that the nuclear areas range from 198 to 625 sq. μ. The difference in volume between the large and medium-sized nuclei is greater than the projected areas suggest, since the large prophase nuclei are thicker than the smaller ones, as shown by the fact that in the former it is possible to focus separately on the nuclear membrane and internally at the level of the nucleoli.

TABLE 2. *Data from 19 Records of Mitosis in Cells of Mouse Spleen*

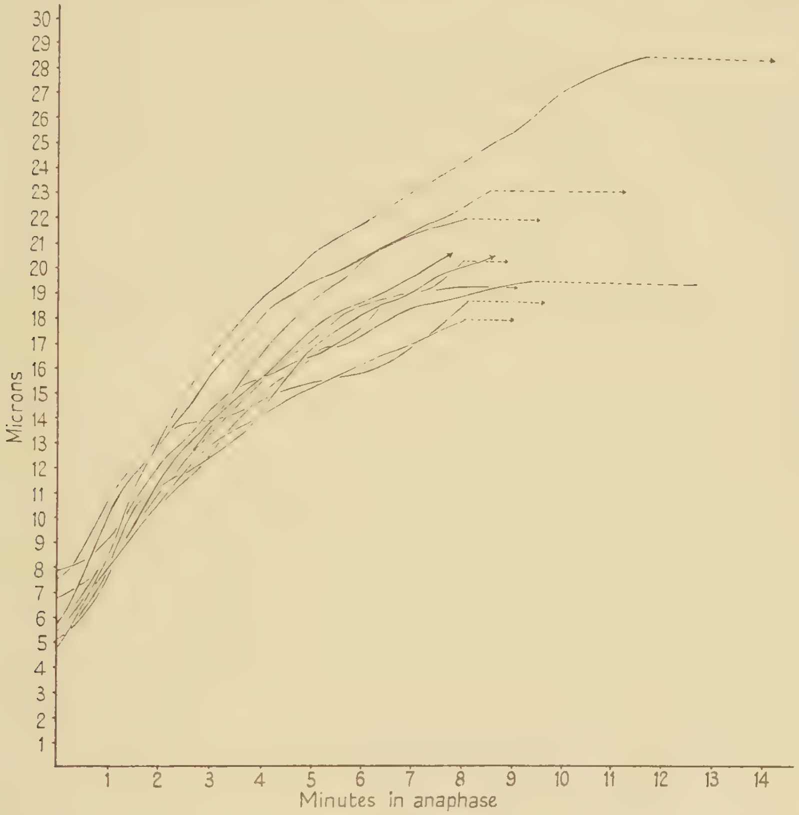
The area of the nucleus in prophase at the beginning of the record and the duration of each phase of division is given.

<table>
<tr><th rowspan="2"><i>Record no.</i></th><th rowspan="2"><i>Nuclear area in prophase in square microns</i></th><th colspan="14"><i>Time of phases in minutes</i></th><th rowspan="2"><i>Type of cell</i></th></tr>
<tr><th colspan="2"><i>Sequence begins in prophase</i></th><th colspan="2"><i>Nucleoli and membrane go</i></th><th colspan="2"><i>Metaphase begins</i></th><th colspan="2"><i>Anaphase begins</i></th><th colspan="2"><i>Cleavage furrow appears</i></th><th colspan="2"><i>Cleavage ends</i></th><th colspan="2"><i>Daughter nucleoli evident</i></th></tr>
<tr><td>1</td><td>198</td><td></td><td colspan="2">13·2</td><td colspan="2">2·1</td><td colspan="2">15·3</td><td colspan="2">5·0</td><td colspan="2">3·8</td><td colspan="2">9·3</td><td></td><td rowspan="10">Medium mononucleate</td></tr>
<tr><td>2</td><td>215</td><td></td><td colspan="2">5·9</td><td colspan="2">5·8</td><td colspan="2">11·6</td><td colspan="2">4·4</td><td colspan="2">4·1</td><td colspan="2">9·7</td><td></td></tr>
<tr><td>3</td><td>229</td><td></td><td colspan="2">4·0</td><td colspan="2">4·1</td><td colspan="2">19·8</td><td colspan="2">3·9</td><td colspan="2">5·6</td><td colspan="2">13·8</td><td></td></tr>
<tr><td>4</td><td>249</td><td></td><td colspan="2">12·8</td><td colspan="2">5·8</td><td colspan="2">20·0</td><td colspan="2">3·5</td><td colspan="2">5·5</td><td colspan="2">24·4</td><td></td></tr>
<tr><td>5</td><td>255</td><td></td><td colspan="2">5·9</td><td colspan="2">5·8</td><td colspan="2">11·6</td><td colspan="2">4·4</td><td colspan="2">4·1</td><td colspan="2">9·7</td><td></td></tr>
<tr><td>6</td><td>270</td><td></td><td colspan="2">30·0</td><td colspan="2">5·5</td><td colspan="2">18·0</td><td colspan="2">4·8</td><td colspan="2">4·8</td><td colspan="2">20·4</td><td></td></tr>
<tr><td>7</td><td>272</td><td></td><td colspan="2">19·0</td><td colspan="2">10·0</td><td colspan="2">7·4</td><td colspan="2">6·6</td><td colspan="4">← 12·0 →</td><td></td></tr>
<tr><td>8</td><td>296</td><td></td><td colspan="2">19·8</td><td colspan="2">13·3</td><td colspan="2">5·7</td><td colspan="2">5·3</td><td colspan="2">2·4</td><td colspan="2">15·6</td><td></td></tr>
<tr><td>9</td><td>315</td><td></td><td colspan="2">21·4</td><td colspan="2">6·6</td><td colspan="2">12·0</td><td colspan="2">5·0</td><td colspan="2">4·0</td><td colspan="2">20·3</td><td></td></tr>
<tr><td>10</td><td>333</td><td></td><td colspan="2">18·8</td><td colspan="2">4·4</td><td colspan="2">9·4</td><td colspan="2">5·4</td><td colspan="2">8·7</td><td colspan="2">26·3</td><td></td></tr>
<tr><td>11</td><td>420</td><td></td><td colspan="2">5·1</td><td colspan="2">4·4</td><td colspan="2">18·6</td><td colspan="2">4·9</td><td colspan="2">5·4</td><td colspan="2">40·0</td><td></td><td rowspan="6">Large mononucleate</td></tr>
<tr><td>12</td><td>451</td><td></td><td colspan="2">2·8</td><td colspan="2">6·5</td><td colspan="2">15·5</td><td colspan="2">5·7</td><td colspan="2">6·5</td><td colspan="2">13·0</td><td></td></tr>
<tr><td>13</td><td>465</td><td></td><td colspan="2">21·0</td><td colspan="2">11·1</td><td colspan="2">14·0</td><td colspan="2">7·7</td><td colspan="2">6·8</td><td colspan="2">4·2</td><td></td></tr>
<tr><td>14</td><td>465</td><td></td><td colspan="2">. .</td><td colspan="2">8·2</td><td colspan="2">3·9</td><td colspan="2">6·4</td><td colspan="2">7·3</td><td colspan="2">11·2</td><td></td></tr>
<tr><td>15</td><td>575</td><td></td><td colspan="2">8·0</td><td colspan="2">4·3</td><td colspan="2">>14·8</td><td colspan="2">. .</td><td colspan="2">. .</td><td colspan="2">. .</td><td></td></tr>
<tr><td>16</td><td>625</td><td></td><td colspan="2">37·8</td><td colspan="2">. .</td><td colspan="2">. .</td><td colspan="2">. .</td><td colspan="2">. .</td><td colspan="2">. .</td><td></td></tr>
<tr><td>17</td><td>330 (both)</td><td></td><td colspan="2">10·6</td><td colspan="2">9·3</td><td colspan="2">17·5</td><td colspan="2">6·1</td><td colspan="2">9·2</td><td colspan="2">24·2</td><td></td><td>Binucleate with cleavage</td></tr>
<tr><td>18</td><td>384 (both)</td><td></td><td colspan="2">20·0</td><td colspan="10">← 103·0 →</td><td></td><td rowspan="2">No cleavage</td></tr>
<tr><td>19</td><td>443 (both)</td><td></td><td colspan="2">3·0</td><td colspan="2">6·0</td><td colspan="2">35·0</td><td colspan="6">← 39·0 →</td><td></td></tr>
</table>

The following observations show that a difference in nuclear size between two cells of the same culture may persist in their descendants through several generations. As already stated, one of the low-power ciné records was analysed exhaustively and a few cell families were traced through several mitoses. The nuclear areas at prophase were measured throughout two of these lineages; the parent of one family was a medium-sized mononucleate reticular cell, and that of the other a binucleate cell of the same type, to which reference has already been made (p. 366), and in which the two interphase nuclei fused in late prophase.

Selected frames of the film were projected at a total magnification of × 600, and the outlines of the nuclei were traced on squared paper. The data thus obtained are given in Text-fig. 1, and show clearly that the descendants of the mononucleate cell all have smaller nuclei than those of the binucleate element.

The results obtained from the measurements of living nuclei further support the view that the medium-sized nuclei are approximately diploid and that the large ones are polyploid. Probably there is considerable variation in chromosome number between the precisely diploid and the polyploid states.



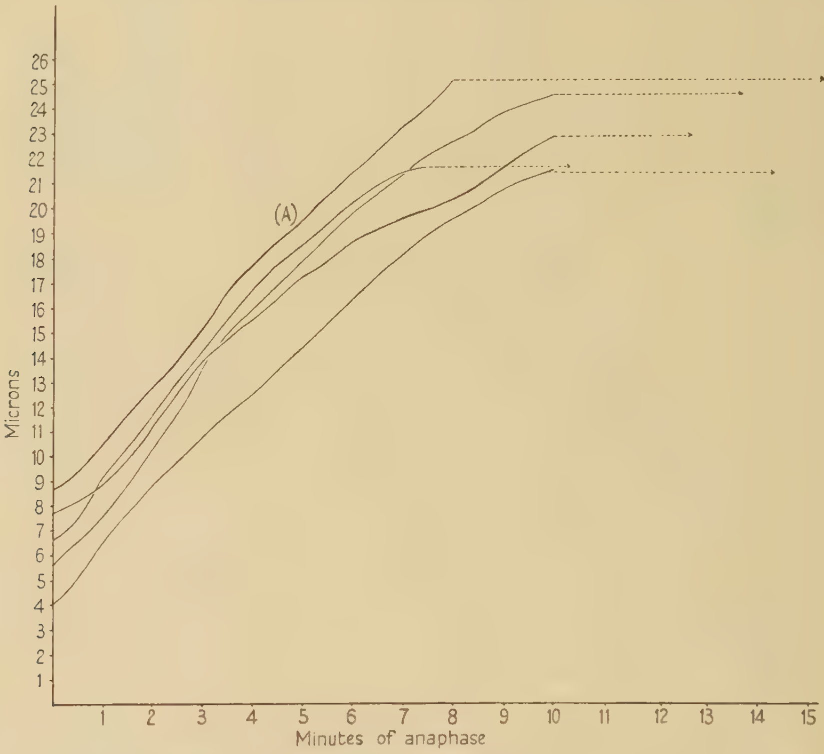
Text-fig. 4. Anaphase curves of a group of ten cells with medium-sized nuclei. The dotted extension of the curve is continued to the point of cleavage of the cell and indicates that the chromosome groups became indistinct in early reconstruction before this point.

The combined areas of the nuclei of binucleate cells in prophase in Table 2 is intermediate between the areas of the nuclei in the two groups of mononucleate cells. It is possible that the nuclei of binucleate cells are generally aneuploid.

III. *Anaphase Movement*

The rate of movement of the chromosomes during anaphase has been measured from the film records, and the results are plotted in Text-figs. 4 and 5. The distance between the daughter chromosomes is measured from the axial centromeres, i.e. from the surfaces of the chromosome groups which face the spindle poles. The duration of the cytoplasmic cleavage is indicated in Text-figs. 4 and 5; after this point nuclear reconstruction had begun, so that the distance measured no longer represents that between the centromeres.

Text-fig. 4 refers to anaphase movement in a group of ten cells with prophase nuclei of medium size. Text-fig. 5 shows the anaphase curves of four cells with large, presumably polyploid nuclei and of one binucleate cell. A comparison of the two figures indicates that the rate of movement in the normal and in the large cells falls within the same range; this implies that the spindle is the same size in both groups, a conclusion that is supported by direct measurements of the distance between the spindle poles. Cleavage of the cytoplasm proceeds more slowly in polyploid and binucleate cells than in diploid elements, probably owing to the larger size of the former.



TEXT-FIG. 5. Anaphase curves of a binucleate cell (A), and of a group of four large mononucleate cells. The dotted lines have the same significance as in Text-fig. 4.

The anaphase curves in the mouse-tissue cultures are similar in shape to those of amphibian cells *in vitro* (Hughes and Preston, 1949). In both there is first a sigmoid inflexion, and the maximum velocity is reached 2–3 minutes from the beginning of anaphase, after which it steadily declines. From the curves of diploid anaphase (Text-fig. 4), an average curve was calculated giving a maximum chromosome velocity of 1·8μ per minute; in *Rana* and *Xenopus* the maximum velocity is about 2·0μ per minute. The final distance between the daughter chromosome groups is similar in *Mus*, *Rana*, and *Xenopus*. The period from the beginning of anaphase to the end of cleavage in *Mus* is intermediate between that in *Rana* and *Xenopus*.

In the chick (Hughes and Swann, 1948) the initial sigmoid inflexion is very small and the daughter chromosomes attain their maximum velocity of 4μ per minute almost as soon as their movement can be accurately measured. Two possible reasons may be suggested for these differences between anaphase movement in the chick and in other vertebrates. The tearing apart of the long chromosome arms is amphibian and mouse mitosis may exert a drag on the centromeres at early anaphase and thus retard their motion; alternatively the spindle may be a better orientated structure in chick cells than in those of other animals. It is unlikely that the maximum rate of anaphase movement is governed by the size of the chromosomes, because in Amphibia, chromosomes of widely different lengths move together with their centromeres ranged on a regular arc.

IV. *Duration of Mitotic Phases*

The duration of each phase of division was calculated from the ciné records and compared with measurements made from direct observation of the living cells during photography. The results are expressed in Table 2.

In Table 2 the mitotic process is subdivided into six stages which, however, cannot be defined with equal precision. As the cell was already in prophase when photography began, the record of this phase of division is incomplete and the longest period recorded is therefore the nearest approach to the true value. By the disappearance of the nucleoli is meant that stage in prophase when the chromosomes attached to the nucleoli become more conspicuous than the nucleolar material itself; these chromosomes remain associated for some minutes longer, however, and it is probable that the nucleoli do not finally disappear until the chromosomes become free. The beginning of anaphase can be determined to within one or two frames of the film. The end of cleavage is usually sharply marked at the time when equatorial constriction ceases, although an isthmus of variable size may connect the daughter cells for some time. The most difficult point to define is that when the nucleoli and chromocentres first become discrete structures; as described above, the nucleolar material is first distinguishable early in telophase before the chromosomes have completely shed their nucleic acid charge.

In Table 2 the total time occupied by division minus the duration of prophase which has to be estimated (see above) is recorded for nineteen cells which are arranged in three groups according to the type of mitosis which they undergo (see p. 365). In group I, the smaller mononucleate cells, mitosis proceeds faster than in the other groups. There is a larger scatter in the duration of each period in the large mononucleate cells of group II than in the smaller cells. The cycle of chromosome changes is slowest in the non-dividing binucleate cells of group III.

Discussion

Different methods of observation have led to divergent views on the structure of the nucleus during interphase. The doctrine of chromosome continuity between successive divisions, although never doubted, was not securely based on direct observation. Although the earlier cytologists described the structure of the interphase nucleus as they saw it in fixed and stained preparations, in terms of chromosome continuity, their views on nuclear structure were not accepted by students of the living cell. Thus Lewis and Lewis (1924) state that in the nuclei of living cells in tissue culture 'no linin network or chromatin granules are to be seen. These are fixation, coagulation, or precipitation products and do not represent living structures.'

This opinion, which was shared by most of those who worked with living tissue cultures, was based on the fact that when living cells in cultures are examined by ordinary direct illumination or by the dark-field method, only the nucleoli are visible and the ground substance appears optically homogeneous. Moreover, M. H. Lewis showed that living cells could be gelated by treatment with dilute acid which produced first a granulation and then a coarse reticulum in the nucleoplasm; under appropriate conditions this gelation could be reversed, when the granules and reticulum disappeared. These interesting experiments naturally led to the conclusion that similar appearances in fixed preparations were artifacts and should be disregarded.

Use of the phase-contrast method suggests that the structures described by earlier cytologists in fixed cells were only partly artificial. The statement that the ground substance of the living nucleus is optically homogeneous is due to the limitations of the microscopical methods previously available. It is well known that unstained structures are visible under the microscope by virtue of differences in refractive index between them and their surroundings. Both the ordinary bright-field and the dark-field microscopes are insensitive to small gradations of refraction. The dark-field microscope brilliantly reveals sharp discontinuities of structure of which the nuclear membrane is an example; as a result the nuclear membrane scatters the converging rays of the dark-field condenser so much that the fine detail in the nucleus does not deflect enough light to be recognized.

These optical limitations of the bright and dark fields are largely overcome by using the phase microscope. As described above, in the living interphase nucleus studied by this means, three types of structure are visible: (1) the nucleoli, (2) small dark granules, some adherent to the nucleoli and others to the inner surface of the nuclear membrane, and (3) fine threads attached to the small granules; the threads, which are the least conspicuous objects in the living nucleus, are seen best in a young cell which has recently divided. When the same nucleus which has been observed and photographed in life is fixed with osmium tetroxide and stained by Feulgen's method, very little distortion is produced; the small dark granules in the living nucleus are found to correspond with the Feulgen-positive chromocentres, and the delicate threads emerging from them are seen to form part of an intricate mass of chromonemata which in the Feulgen-stained nucleus are seen completely to fill the space between the nucleoli and the nuclear membrane.

In the well-fixed cells composing the thin zone of outgrowth, the filamentous structure in the nuclear ground substance is only revealed by careful scrutiny under the most critical optical conditions. On the other hand, many of the interphase nuclei left sticking to the glass after removal of the explant are less perfectly fixed, owing to the slower penetration of the reagent in this part of the culture, and these often show a fine network resembling the linin reticulum described by earlier workers. In our view, this appearance is caused by the defective fixation of the chromonemata which have become irregularly clotted together instead of being evenly distributed in the nucleus in the normal way. Thus the linin network of the earlier observers may be regarded as a real cytological entity artificially distorted by fixation. Bensley (1933) showed that nuclei fixed by the freeze-drying technique were similar in appearance to those treated with chemical fixatives, and concluded that the structures seen in stained preparations of the nuclei were not mere fixative artifacts.

The structure and cytochemistry of the resting nucleus has recently been investigated by Serra (1947) who concluded that 'mitotic chromosomes usually arise from resting nuclei of the thread type, that is from nuclei in which threads which take up chromatin dyes and give strong Feulgen and arginine reactions are conspicuous'.

The state of the chromosomes in the interphase nucleus has recently been investigated by Ris and Mirsky (1949) who, like the earlier workers on tissue cultures, state that 'In the living interphase nucleus no chromosomal structures are visible' (p. 500). As described above, in the living interphase nuclei of mouse cells grown *in vitro*, phase contrast (not used by Ris and Mirsky) reveals fine threads which we regard as chromonemata; the threads are most distinct in young daughter nuclei (Pl. VII, fig. 34*g* and *k*). In the mature nucleus they are usually best seen near their union with the chromocentres; precisely the same type of structure is visible in the nuclei after fixation with osmium or Maximow's fluid (Pl. II, figs. 3 and 4); sometimes the same chromonema can be identified in a nucleus before and after fixation. In the fixed nucleus, however, the complete system of chromonemata is distinguishable. The threads are faintly Feulgen-positive, and a small amount of desoxyribonucleic acid may have been deposited on them during fixation, as described by Ris and Mirsky for fixatives other than osmium. As recorded above, in the mouse cells during telophase each chromosome rapidly swells and thus enters the 'extended state' of Ris and Mirsky, but in our material the chromonemata became clearly visible during the swelling process (see p. 363).

In certain plants the number of Feulgen-positive granules or chromocentres in the nucleus is approximately the same as that of the chromosomes (Wilson, 1925). Overton (1909) named them 'prochromosomes' and suggested that they were aggregates of residual chromatin around which the chromosomes were formed early in prophase. Heitz (1928) showed that the prochromosomes were segments of the mitotic chromosomes, which retained the full charge of nucleic acid in interphase. These segments were called 'heterochromatic'. Recent literature on the nature and function of 'heterochromatin' has been reviewed by Schultz (1947). The chromocentres of human tumour cells have been studied by Koller (1947) and those of mouse carcinomata by Biesele (1944*b*).

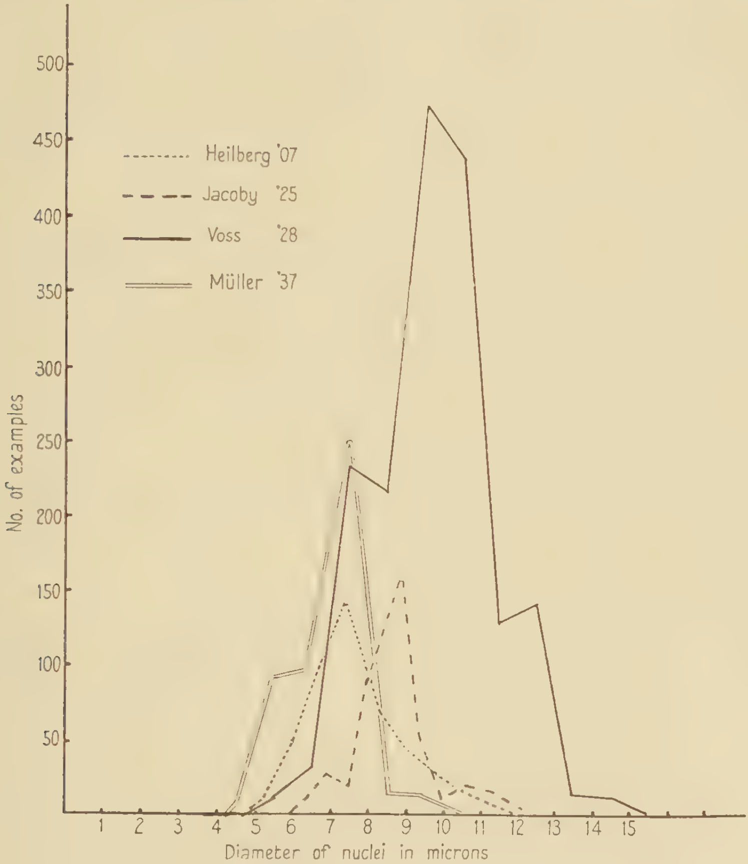
A striking feature of the chromocentres is their apparent adhesiveness; they are always attached to the surface of either the nucleoli or the nuclear membrane. This characteristic, which has also been noted by Vanderlyn (1946), is very conspicuous in the living mouse nuclei viewed with the phase microscope. There is almost no depth of focus with the phase microscope, so that it is possible to focus separately on a number of planes even in so thin a structure as the nucleus of a tissue-culture cell. On the surface of the nuclear membrane the mitochondria are seen, and a very slight movement of the fine adjustment reveals the chromonemata and chromocentres adherent to the inner surface of the membrane. In a large polyploid nucleus in early prophase, the focus can then be shifted to the level of the nucleoli, to which other chromocentres are attached (Pl. VI, fig. 33*b* and *c*). This distribution of the chromocentres is less obvious in fixed and stained preparations, as fixation and dehydration shrink the nucleus in a direction vertical to the coverslip. The nucleolar chromocentres remain stuck to the dwindling nucleoli late in prophase. The chromonemata become well charged with desoxyribonucleic acid before the nucleoli disappear, so that towards the end of prophase chromosomes radiate from the nucleoli in the manner described by Lewis (1940).

If the heterochromatic segment of the chromosomes is adjacent to the centromere, the attachment of the chromocentres to the nuclear membrane or nucleolus might be regarded as equivalent to the attachment of the chromosomes to the spindle. Unfortunately, however, we have been unable to find conclusive evidence as to which end of the chromosome is occupied by the heterochromatic segment.

As described above, the so-called euchromatic region of the chromosome, which is adjacent to the heterochromatic segment, is reduced in interphase to the residual chromonema by the loss of most of the nucleic acid charge. It is usually believed that this process is accompanied by an uncoiling of the chromonema: 'the change from metaphase to resting nucleus consists spatially in an uncoiling and repacking of the chromosome thread' (Darlington, 1937, p. 34). Conversely, in prophase the chromonema is thought to become coiled as it acquires its charge of nucleic acid. In our material the chromosomes are too small for any coiling or uncoiling to be clearly distinguishable, but, as already stated, the mouse chromosomes in prophase often have a finely banded appearance which may represent the gyres of a fine coil.

A relationship between nuclear size and chromosome content was first noted by Boveri (1905) in haploid, diploid, and tetraploid sea-urchin embryos.

His observations were confirmed by Balzer (1910) and Herbst (1912), and similar conclusions were reached by Artom (1928) who studied diploid and tetraploid races of *Artemia salina*. In Amphibia, heteroploidy has been investigated in great detail and the literature has recently been reviewed by Fankhauser (1945). Heteroploid division has also been described in normal mammalian cells. It has been seen in human spermatogonia and spermatocytes (Painter, 1923), in the rat liver (Biesele, 1944*a*) and in the embryonic tissue of the mouse (Howard, personal communication).



TEXT-FIG. 6. Histograms of the nuclear diameter of cells of the mouse liver, extracted from the data given by the authors indicated.

The interphase nucleus of normal mammalian cells vary considerably in size and several authors, notably Jacoby (1925, 1935), have attempted to show that in distribution curves of nuclear sizes, there are modes which correspond to simple multiples of nuclear volume (ibid.). Nuclear size has been studied in the liver of the mouse by Heiberg (1907), Jacoby (1925), Voss (1928), and Müller (1937). The distribution curves of nuclear diameters derived from the data given by these authors are plotted in Text-fig. 6; it is hardly possible to identify the modes of these curves with degrees of heteroploidy. In the rat liver also, nuclear sizes have been investigated by Jacoby (1935) and by Beams and King (1942), but the curves obtained in these two studies also fail to agree.

Biesele (1944*a*) made chromosome counts in rat liver and found a slight preponderance of diploid over tetraploid nuclei in both the normal and regenerating tissue; the proportion of octoploid nuclei was much smaller. He also observed irregularities in chromosome number which were not exact multiples of the haploid number and which are termed aneuploid. If aneuploid cells are fairly numerous, their presence must contribute to the difficulty of interpreting data on nuclear size. Our own observations suggest that aneuploid nuclei are fairly common in mouse tissue cultures.

Distribution curves of nuclear size can only indicate that a cell population is not uniform and more direct evidence is needed before heterogeneity in volume can be attributed to polyploidy. Such evidence was obtained by Beams and King (1942) in the regenerating liver of the rat. These authors concluded that tetraploid nuclei were formed by the mitosis of binucleate cells. Their description of this process, based on the study of stained preparations, is exactly confirmed by our own observations on living mouse cells *in vitro*. They also describe the formation of a cell with two tetraploid nuclei from one with two diploid nuclei, by mitosis without cleavage; this, too, we have observed in life (see p. 366). Formerly, nuclei of double size in the liver were thought to arise by an amitotic process (Jacoby, 1925, 1935; Müller, 1937), a view which was discarded by Beams and King; we ourselves have never seen amitotic division in vertebrate tissue cultures.

Our own observations on the formation of polyploid cells in tissue cultures were made before we had read the paper by Beams and King. That similar results should have been obtained *in vivo* shows that the variation in the mitotic process whereby polyploid cells are produced is not an abnormality peculiar to tissue cultures. It would be interesting to know whether the origin of polyploid from binucleate cells is a general phenomenon in mammalian tissues. Wherever binucleate and large mononucleate cells occur together, it may well be suspected that the latter originate from the former.

In the Insecta polyploidy is achieved by a quite different process, viz. 'endomitosis'. Thus, in the nurse cells of the ovary of *Drosophila*, the chromosomes split and double within the nuclear membrane during prophase (Painter and Reindorp, 1939). Biesele (1944*b*) suggested that in the nuclei of tumour cells chromosomes may double their size by an endomitotic process.

We are indebted to the Royal Society for a grant to cover the cost of additional plates, and to the Medical Research Council and the British Empire Cancer Campaign by whom the expenses of the research were defrayed. We also wish to thank our assistant, Mr. L. J. King, for his help with the tissue cultures.

SUMMARY

1. Mitosis has been investigated in cultures of infant mouse tissues, in both living and fixed material.

2. Phase microscopy combined with serial photography was used to study the living cell.

3. The formation of chromosomes has been traced in the living cell from the intermitotic chromonemata and heterochromatic chromocentres.

4. Dividing cells can be recognized early in prophase and the duration of each phase of mitosis has been measured.

5. Curves of anaphase movement have been plotted; they are sigmoid as in amphibian cells.

6. The reconstruction of the daughter nuclei has been studied in detail in both living and fixed cultures. The chromosomes swell, then begin to lose their nucleic acid charge and to unravel into a web of fine threads. The nucleolar material can be identified in the living cell early in telophase; it appears as a diffuse system of lumps and strands occupying most of the nucleus and its direct transformation into the interphase nucleoli was followed in the film records.

7. The origin of polyploid nuclei by the mitosis of binucleate cells has been demonstrated.

8. Analysis of the film records showed that the average length of the intermitotic period is 12 hours.

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DESCRIPTION OF PLATES

Plate I

All × 2,800

Fig. 1. Cell in early anaphase (spleen culture).
(*a*) By phase contrast, living: note the pattern of mitochondria around spindle.
(*b*) By phase contrast, fixed in OsO_4; the chromosomes are invisible, but the outline of the spindle is seen.
(*c*) Stained with the Feulgen reagent.

Fig. 2. Cell in interphase (spleen culture).
(*a*) By phase contrast, living: within the nucleus the small dark granules are chromocentres and the larger bodies are nucleoli.
(*b*) By phase contrast, fixed. The chromocentres are almost invisible.
(*c*) Stained with the Feulgen reagent. Note the very close correspondence of the chromocentres and nucleoli in (*a*) and (*c*).

Plate II

Nuclei in interphase, and in normal diploid mitosis, × 2,800; all except Fig. 3 are Feulgen preparations.

Fig. 3. Intermitotic nucleus (spleen culture). Note the chromonemata in the ground substance. Haematoxylin.

Fig. 4. Intermitotic nucleus (kidney culture), showing nucleoli, chromocentres, and chromonemata.

Fig. 5. Early prophase (spleen culture). The chromonemata are more distinct and the chromocentres smaller and more numerous than at interphase.

Fig. 6. Slightly later prophase (kidney culture). Nucleoli are still present.

Fig. 7. Later prophase (kidney culture). The nucleoli have disappeared. Note the terminal heterochromatic granules (*h*) on the chromosomes.

Fig. 8. End stage of prophase in spleen cell, just before collapse of nuclear membrane. The chromosomes are now obviously double.

Fig. 9. 'Prometaphase' in kidney cell. The nuclear membrane has gone and the chromosomes have contracted. The chromosomes lie in a plane parallel to the coverslip which is rare in mouse-tissue cultures.

Fig. 10. Metaphase in spleen cell. The chromosomes are not yet equatorially arranged.

Fig. 11. Full metaphase in spleen cell.

Fig. 12. Early anaphase in spleen cell.

Fig. 13. Telophase in spleen cell. The chromosomes form a tight mass.

Fig. 14. Daughter nucleus in reconstruction (heart culture).

Fig. 15. Daughter nucleus in slightly later reconstruction (spleen culture). Some of the chromosomes appear double.

PLATE III

Tetraploid and binucleate cells in interphase and mitosis. × 2,800. All Feulgen preparations, except Fig. 21.

Fig. 16. Tetraploid nucleus in interphase (spleen culture).

Fig. 17. Binucleate cell in interphase (kidney culture).

Fig. 18. Tetraploid cell in very early prophase (kidney culture).

Fig. 19. Tetraploid cell in prophase (kidney culture). The nucleoli have nearly disappeared.

Fig. 20. Binucleate cell in late prophase (spleen culture).

Fig. 21. Tetraploid cell at the end of prophase (spleen culture). Haematoxylin. The nuclear membrane has disappeared, and the chromosomes are crowding round the two opposite poles of the spindle (compare Pl. VIII, fig. 37*b*).

PLATE IV

All Feulgen preparations, × 2,800, except Fig. 30.

Fig. 22. Tetraploid metaphase (spleen culture). Compare with Pl. II, fig. 11.

Fig. 23. Early anaphase of a tetraploid cell (kidney culture). Compare with Pl. II, fig. 12.

Fig. 24. Telophase (spleen culture). The daughter nuclei remain within one cell.

Fig. 25. Reconstruction of a normal diploid daughter nucleus (spleen culture) at a later stage than in Pl. II, fig. 15. Note the fine transverse filaments connecting the chromosomes.

Fig. 26. Reconstruction of a tetraploid daughter cell at the same stage as in Fig. 25 (kidney culture).

Fig. 27. Late stage in the reconstruction of a diploid nucleus (kidney culture). The nucleoli and chromocentres are now distinct.

Fig. 28. Late reconstruction stage of a tetraploid nucleus (kidney culture). Compare with Fig. 27.

Fig. 29. Late reconstruction stage in a binucleate cell (spleen culture).

Fig. 30. Stages in the formation of a binucleate cell in a spleen culture, photographed by phase contrast at low power; all enlarged to × 220.

(*a*) The upper cell is approaching the end of interphase.

(*b*) 2½ hours later. Cell in anaphase.

(*c*) 12 min. later than (*b*). Cleavage apparently complete.

(*d*) 28 min. later than (*c*). Daughter cells flattening, but still connected by a strand.

(*e*) 15 min. later than (*d*). They reunite to form one binucleate cell.

PLATE V

Fig. 31. Mitosis in a diploid cell (spleen culture). Phase contrast enlargements, all × 1,500, from frames of 16 mm. film. Duration of complete record: 74 min.

(*a*) Prophase. Nucleoli still present but chromosomes developing.

(*b*) 3·7 min. later than (*a*). The nuclear membrane is shrinking, and is indented by the spindle asters above and below.

(*c*) 6·3 min. later than (*b*). Early metaphase. The spindle is outlined by mitochondrial material.

(*d*) 9·7 min. later than (*c*).

(*e*) 3·4 min. later than (*d*). The split in the metaphase chromosomes is clearly visible.

(*f*) 0·9 min. later than (*e*). The beginning of anaphase.

(*g*) 3·1 min. later than (*f*). Mid-anaphase. The interzonal region of the spindle is being constricted as is shown by the inward movement of mitochondria.

(*h*) 4·5 min. later than (*g*). Orientated mitochondria are seen in the interzonal region. The groups of daughter chromosomes are closely packed.

(*i*) 2·5 min. later than (*h*). Cleavage nearly complete and the groups of daughter chromosomes are swelling.

(*j*) 9·8 min. later than (*i*). The daughter cells are connected by an interzonal strand (*s*). In the lower daughter the nuclear membrane is visible. The nucleoli are now distinct.

(*k*) 13·0 min. later than (*j*). The young interphase nucleus is increasing in size, and the nucleoli have more contrast.

(*l*) 16·7 min. later than (*k*). Further development of the interphase nucleus.

Plate VI

Fig. 32. Prophase and metaphase of a polyploid cell (spleen culture). Phase-contrast enlargements, all × 1,500, from frames of 16-mm. film. Duration of record: 25·4 min.

(*a*) Prophase. Nucleoli are still present. Compare nuclear size with that in Pl. V, fig. 31*a*.

(*b*) 5·6 min. later than (*a*). Nucleoli are disappearing.

(*c*) 5·9 min. later than (*b*). The asters have deeply indented the nuclear membrane.

(*d*) 1·9 min. later than (*c*). The nuclear membrane has disappeared; the spindle has been formed and chromosomes are migrating towards it.

(*e*) 8 min. later than (*d*). Many of the chromosomes are not yet on the equator of the spindle.

(*f*) 4 min. later than (*e*). The chromosomes are nearer the metaphase arrangement.

Fig. 33. Prophase of a polyploid cell (spleen culture). Phase-contrast enlargements, all × 1,500, from frames of 16-mm. film record. Duration of record: 23 min.

(*a*) A polyploid prophase. The plane of focus is within the nucleus, at the level of the nucleoli.

(*b*) 14 min. later than (*a*). Note the change in shape and arrangement of the nucleoli.

(*c*) Immediately after (*b*). The focus has been changed to the level of the nuclear membrane, on the inside surface of which are many chromosome threads.

(*d*) 8·8 min. later than (*c*). The development of the chromosomes is further advanced.

Plate VII

Fig. 34. Anaphase, telophase, and reconstruction of one daughter nucleus of a polyploid cell in a spleen culture. Phase-contrast enlargements, ×2,530, from frames of 16-mm. film. Duration of complete record: about 5 hours. For description see p. 363. n = nucleolus. *ch* = chromonema.

(*a*) 4 min. after the beginning of anaphase.
(*b*) 2·3 min. later than (*a*).
(*c*) 11·1 min. ,, ,, (*b*).
(*d*) 6·1 min. ,, ,, (*c*).
(*e*) 4·5 min. ,, ,, (*d*).
(*f*) 7 min. ,, ,, (*e*).
(*g*) 19 min. later than (*f*)
(*h*) 60 min. ,, ,, (*g*).
(*i*) 15 min. ,, ,, (*h*).
(*j*) 22 min. ,, ,, (*i*).
(*k*) 24 min. ,, ,, (*j*).

Plate VIII

Fig. 35. Polyploid nucleus in prophase, to show movement of chromosomes (spleen culture). Phase-contrast enlargements, × 1,500, from frames of 16-mm. film. Duration of record: 17 min.

(*a*) Late prophase, with nucleoli gone.

(*b*) 2 min. later than (*a*). Nuclear membrane contracting.

(*c*) 15 min. later than (*b*). Most chromosomes have crowded round the two asters, which lie to the left of the nuclear area. A group of chromosomes at the top remain separate, and are not brought on to the metaphase plate until 25 min. later.

Fig. 36. Mitosis of a binucleate cell (spleen culture). Phase-contrast enlargements, × 1,500, from frames of 16-mm. film. Duration of record: 129 min.

(*a*) Late prophase; nucleoli and nuclear membrane still present.

(*b*) 9 min. later than (*a*). Nuclear membranes have gone, and the two diploid sets of chromosomes come together.

(*c*) 40 min. later than (*b*). Anaphase.

(*d*) and (*e*) 80 min. later than (*c*). The polyploid nucleus of each daughter cell.

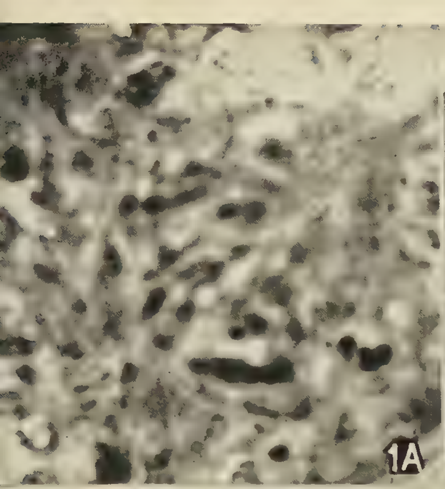
Fig. 37. Mitosis of a binucleate cell, without cleavage (spleen culture). Phase-contrast enlargements, × 1,500, from frames of 16-mm. film. Duration of record: 253 min.

(*a*) Prophase. Nucleoli and nuclear membranes still present.

(*b*) 3 min. later than (*a*). The nuclear membranes have gone and the diploid chromosome sets have come together.

(*c*) 125 min. later than (*b*). Tetraploid daughter nuclei now nearly as big as the parent diploid nuclei and still in the same cell.

(*d*) 125 min. later than (*c*). Rearrangement of the nucleoli in each nucleus.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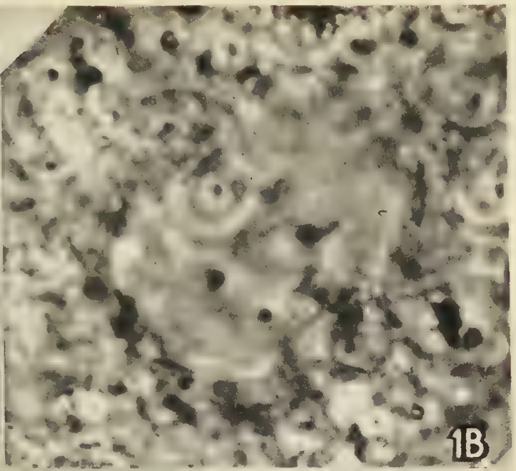
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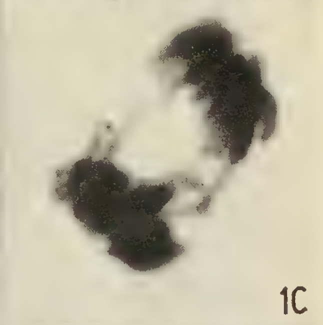
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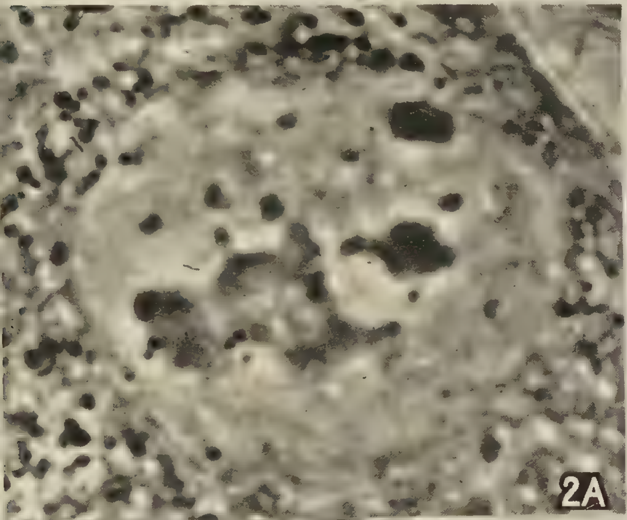
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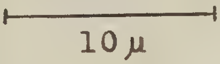

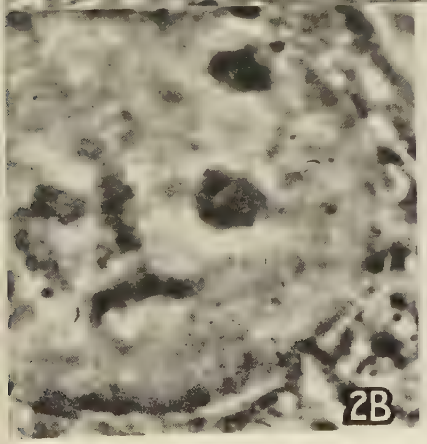
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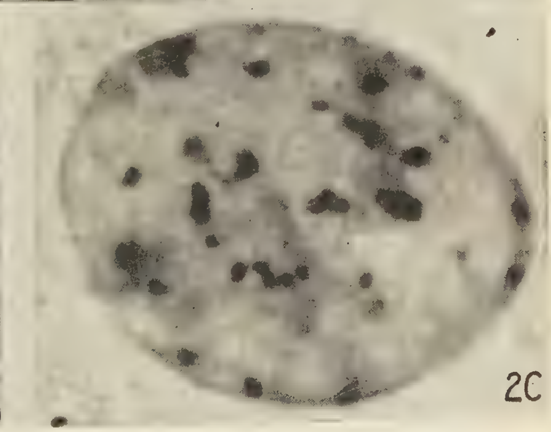
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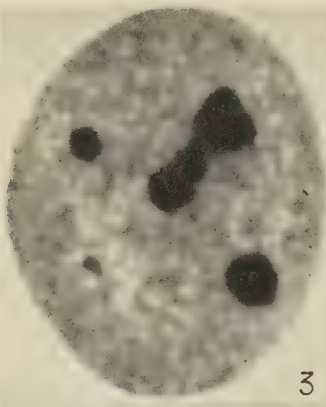
H. B. FELL AND A. F. HUGHES—PLATE I



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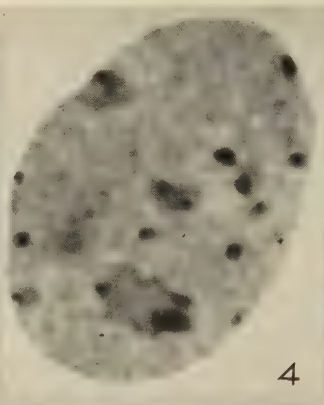

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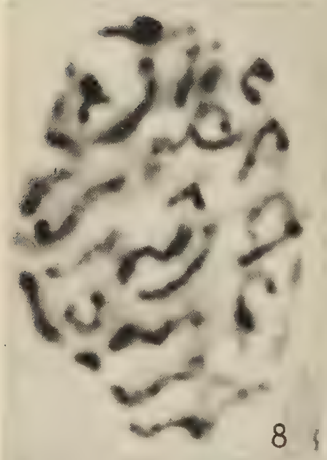

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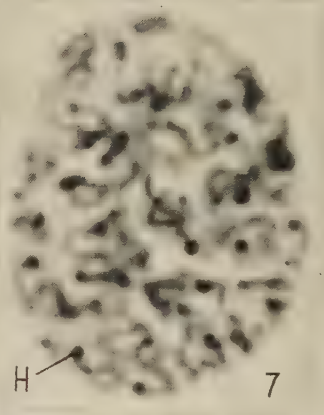

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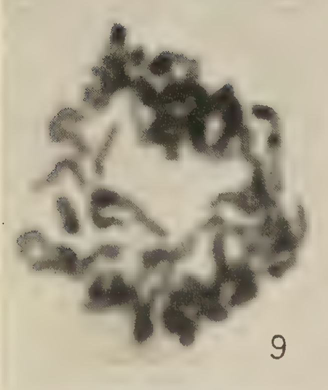

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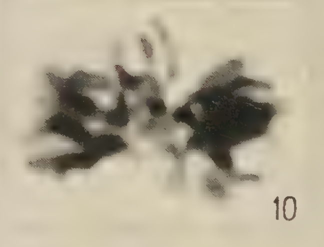

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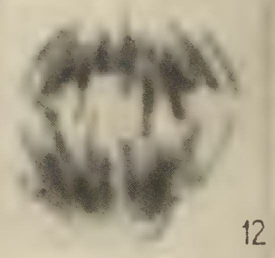

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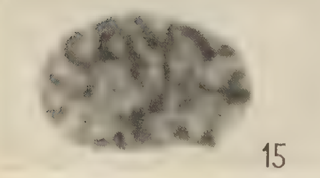

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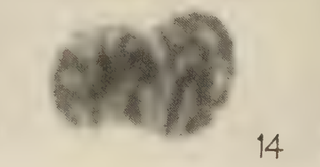

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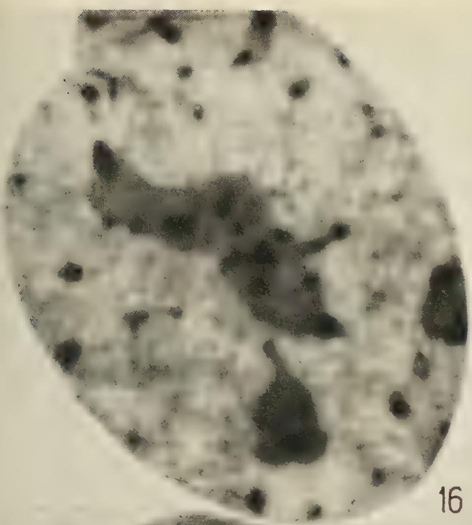
H. B. FELL AND A. F. HUGHES—PLATE II



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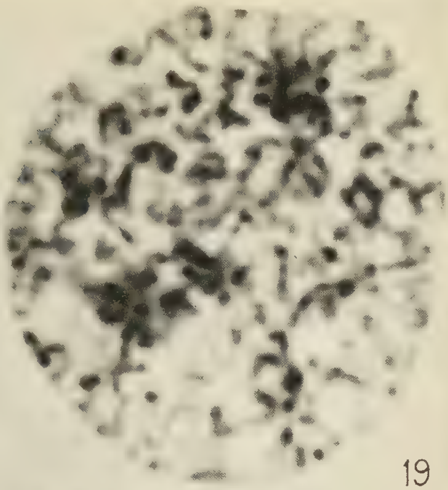

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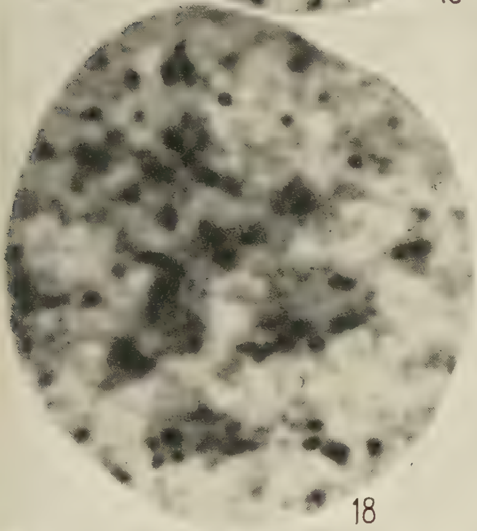

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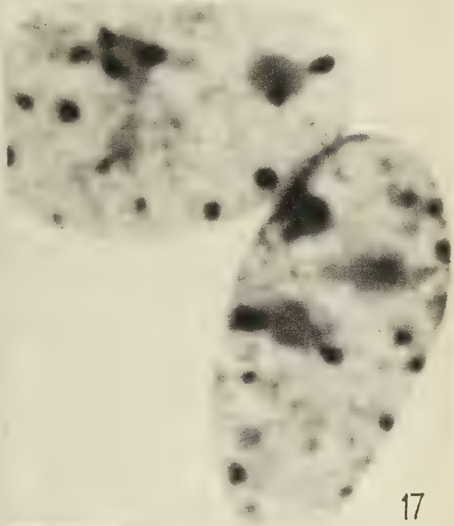

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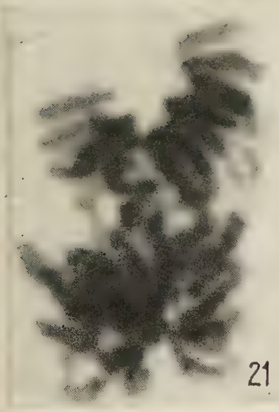

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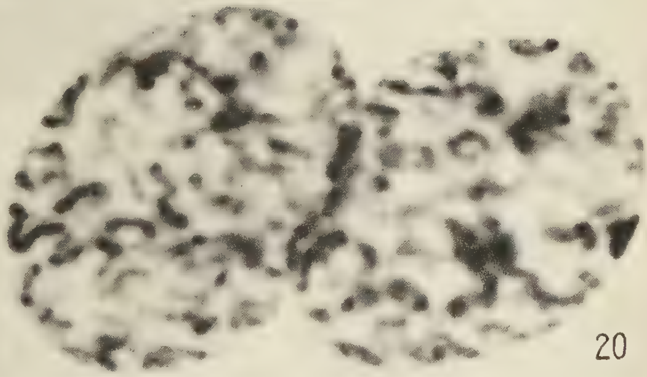

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H. B. FELL AND A. F. HUGHES—PLATE III

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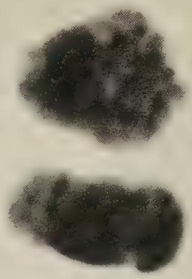
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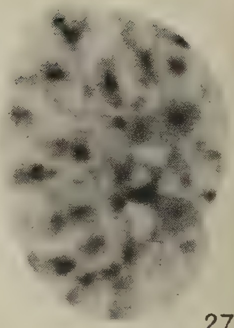
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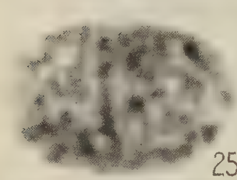
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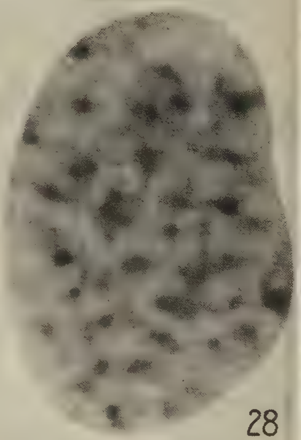
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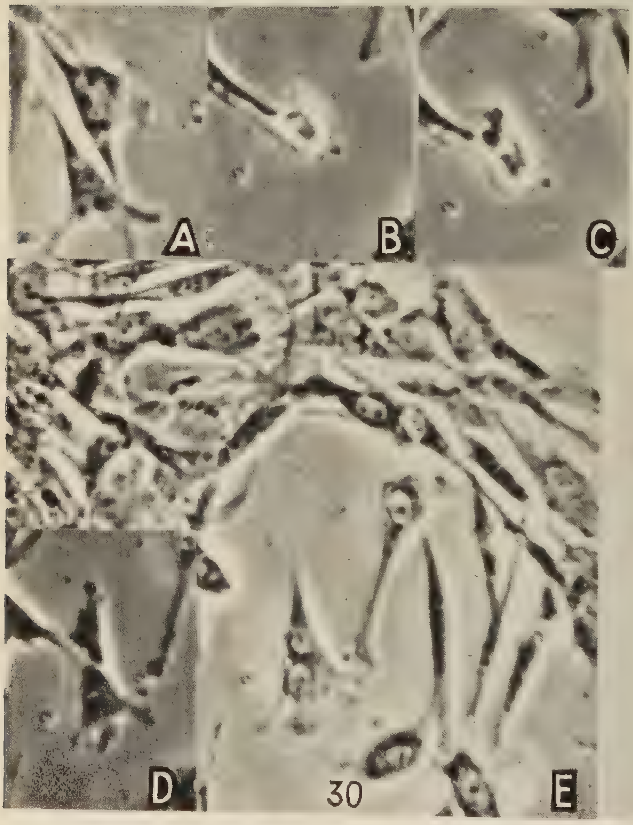

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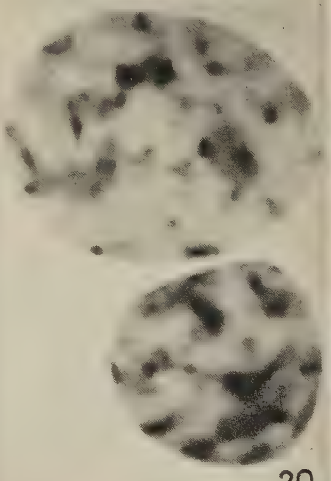
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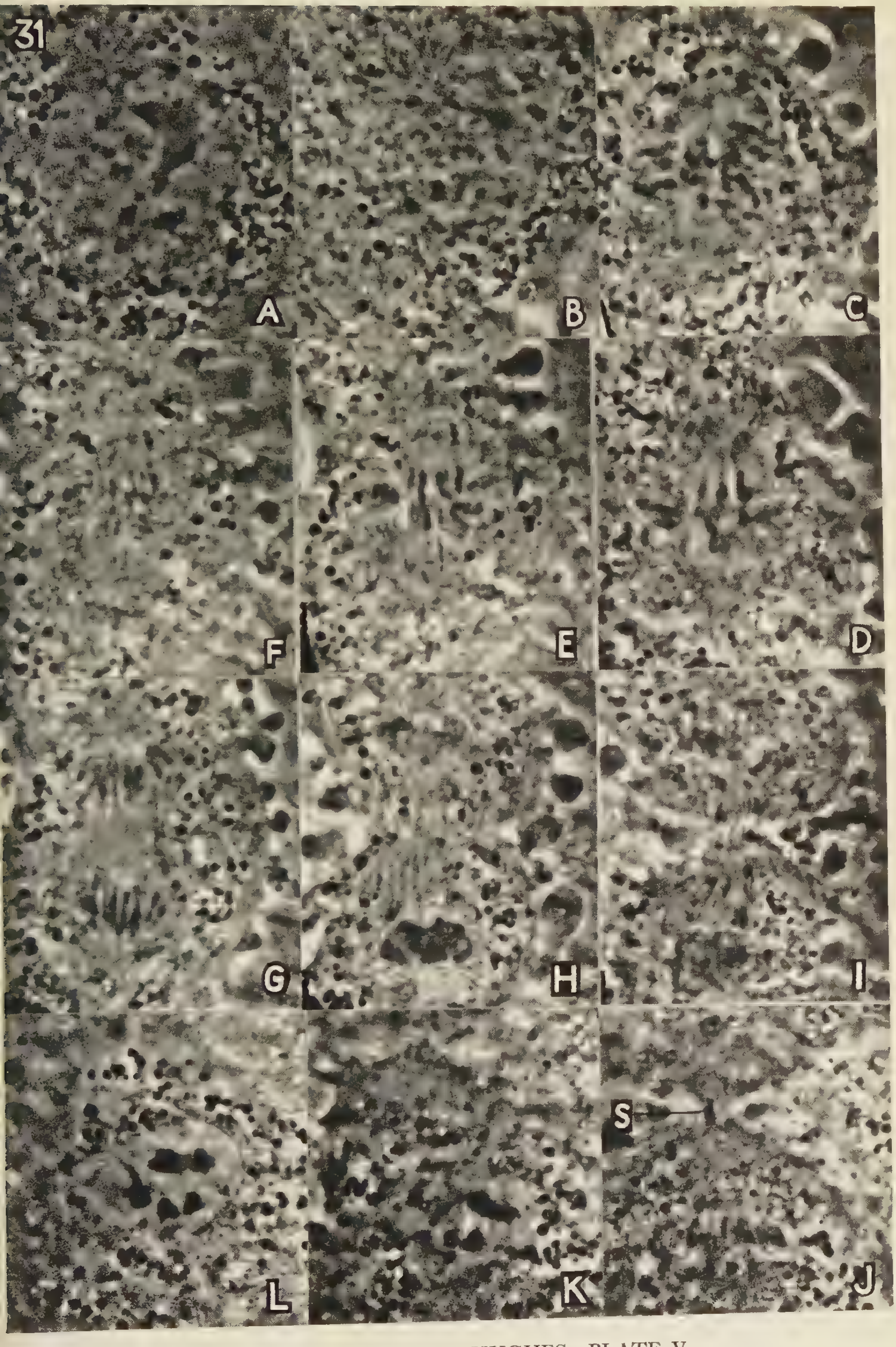
H. B. FELL AND A. F. HUGHES—PLATE IV



H. B. FELL AND A. F. HUGHES—PLATE V

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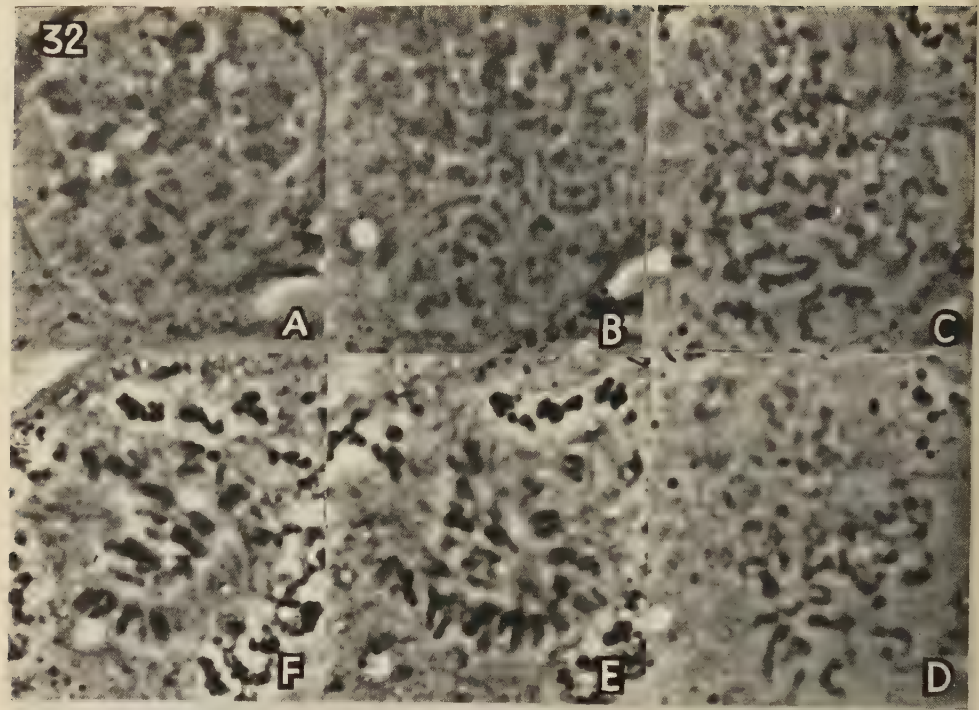

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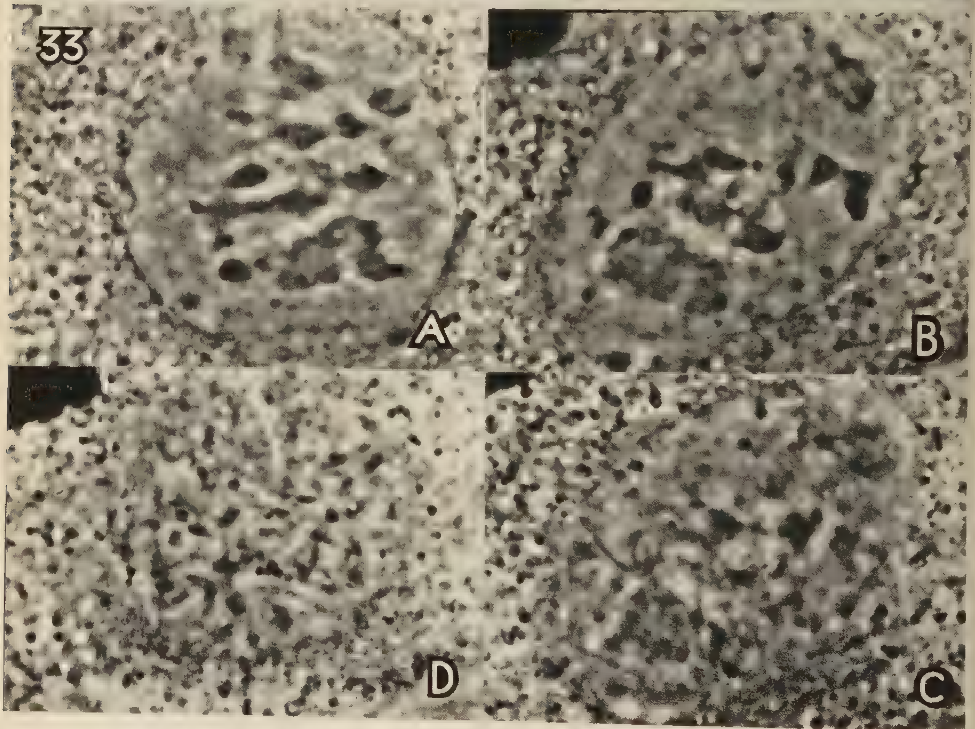
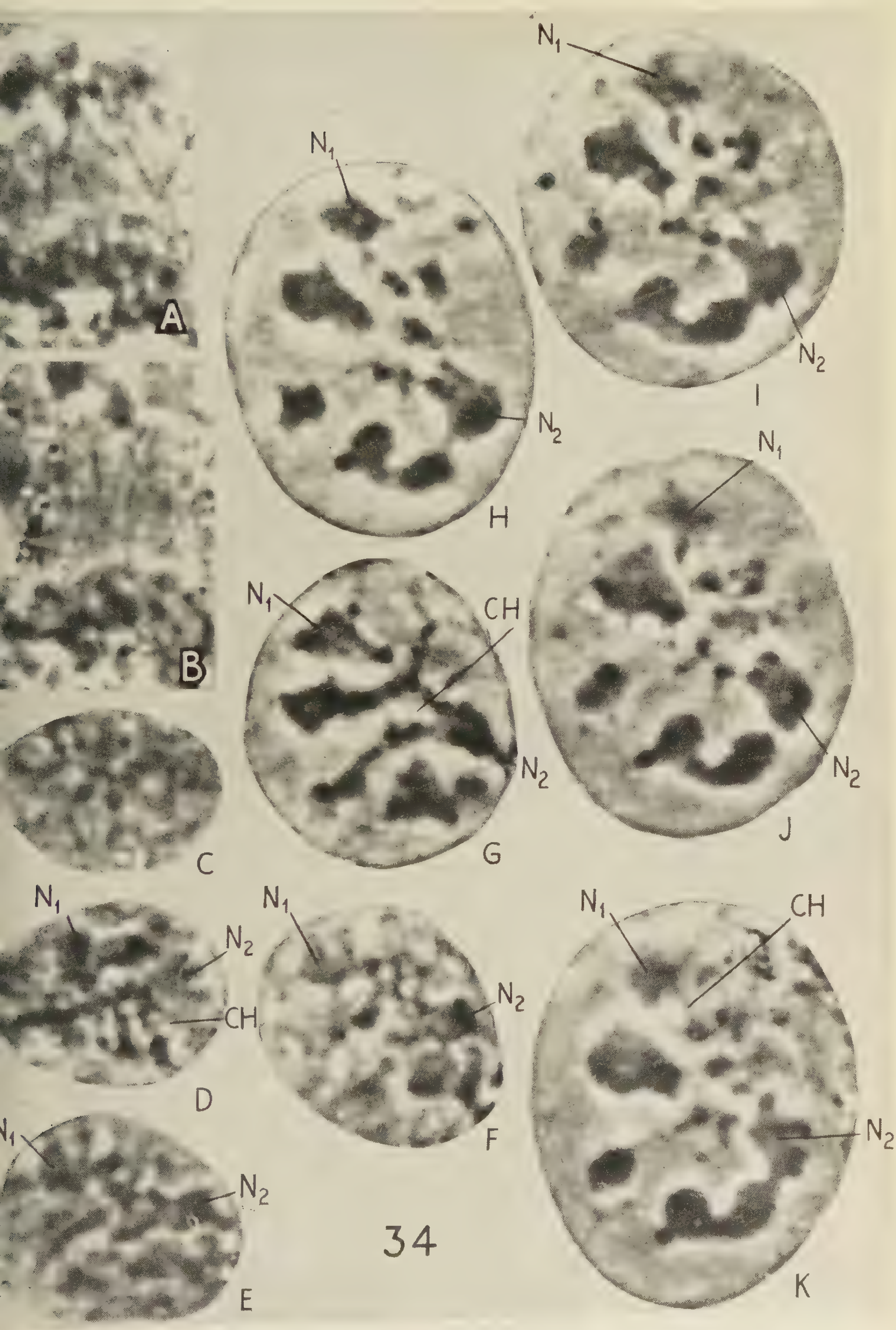
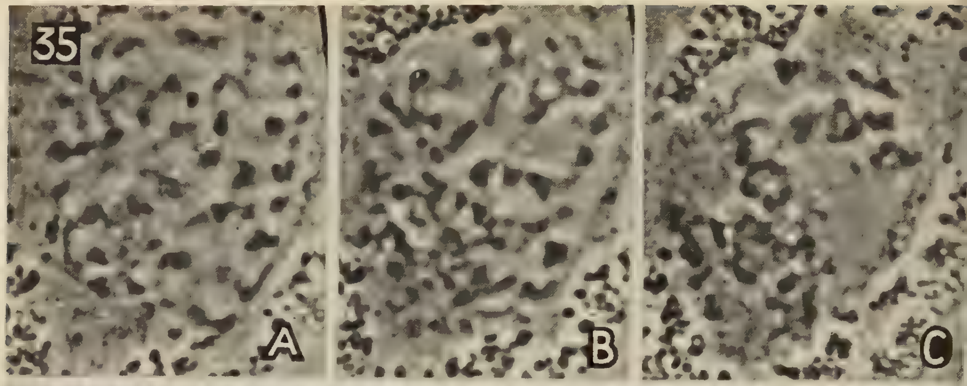


H. B. FELL AND A. F. HUGHES—PLATE VI



H. B. FELL AND A. F. HUGHES—PLATE VII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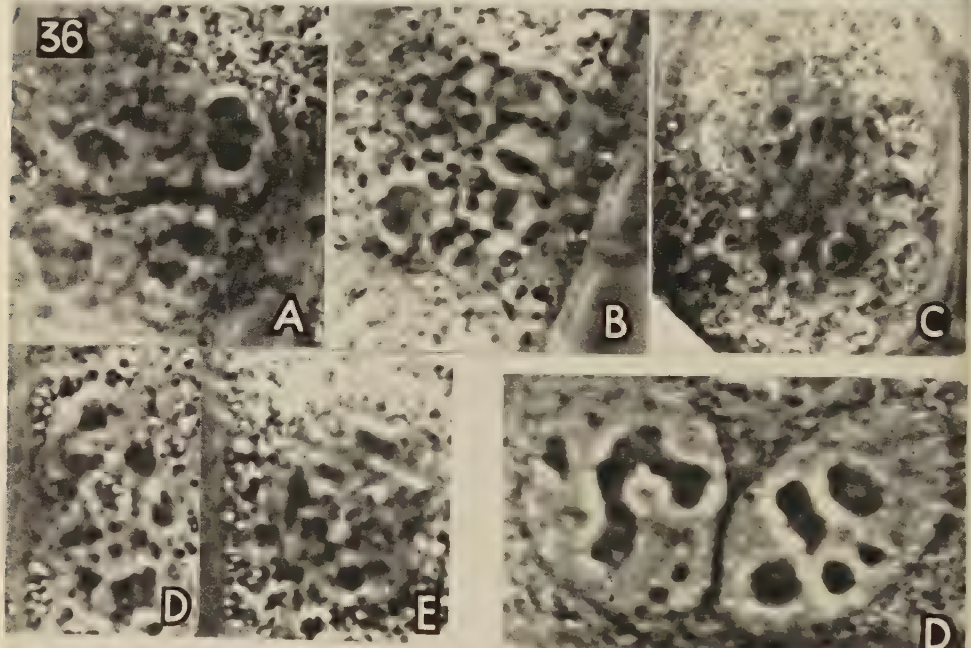
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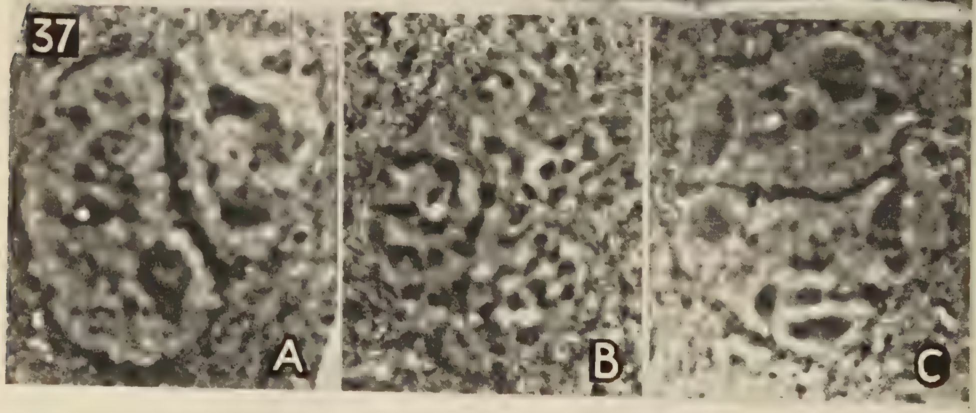
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H. B. FELL AND A. F. HUGHES—PLATE VIII

The Distribution of Alkaline Phosphatase in Relation to Calcification in *Scyliorhinus canicula*

Development of the Endoskeleton

BY

I. JOAN LORCH

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With one Plate

INTRODUCTION

IN teleosts and all higher vertebrates calcification is a preliminary to ossification. But in elasmobranchs calcified cartilage functionally replaces bone. The histology and biochemistry of elasmobranch cartilage have been reviewed by Weidenreich (1930). In spite of the vast literature on the subject, the relation of the calcium salts to the matrix is not fully understood and there is disagreement regarding the presence and amount of calcium phosphate. However, the following points seem established: calcification always starts at the periphery of a piece of cartilage, at a point remote from the cells. Amorphous granules are first formed which later coalesce into an irregular mosaic of crystalline plates. In *Scyliorhinus* the platelets cover the inner and outer surfaces of the vertebral arches and the skull and the total surfaces of the visceral skeleton. The pattern of calcification in the vertebral column of the higher elasmobranchs is so typical that it has been used as a basis of classification (Hasse, 1893). In view of these facts a study of the distribution of alkaline phosphatase in an elasmobranch seemed of interest. Here normal calcification can be studied without the complications of cartilage hypertrophy and resorption and the simultaneous formation of a new protein matrix which always accompany ossification.

Badansky, Bakwin, and Bakwin (1931) showed that alkaline phosphatase exists in elasmobranchs, but drew no correlation between phosphatase activity and calcification. The question was studied in greater detail by Roche and Bullinger (1939). These authors showed that the phosphatase present in the skeleton of both teleosts and elasmobranchs belongs to the Class IA of Folley and Kay (1936); i.e. it is closely similar to that found in mammalian bones. By means of microchemical tests Roche and Bullinger showed that only the calcifying regions of elasmobranch skeletons contained the enzyme, adjacent soft portions being negative. These results as well as a study of phosphatase in relation to the growth of scales and teeth (Roche, Collet, and Mourgue, 1940) led the authors to conclude that in elasmobranchs, as in higher vertebrates, phosphatase is concerned with rapid calcification.

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

No histochemical study of the fish skeleton seems to have been undertaken, nor has phosphatase been studied during the embryonic development of lower vertebrates. It therefore seemed desirable to undertake a systematic study of the histological and cytological distribution of alkaline phosphatase in developing teleost (Lorch, 1949) and elasmobranch embryos. Only the endoskeleton is considered here.

METHODS

Twenty egg-cases and five hatched specimens of *Scyliorhinus canicula* were obtained living from the Marine Biological Laboratory, Plymouth. After removal of egg-case and yolk-sac the specimens were anaesthetized in 10 per cent. ethyl alcohol, measured, and placed into 80 per cent. ethyl alcohol in distilled water. When sufficiently hardened they were divided into a number of blocks according to the size of the fish and fixation was continued up to 24 hours. It was found that when 80 per cent. alcohol in *sea* water was used as fixative, considerable destruction of phosphatase occurred. The reason for this is not clear. The heads of three of the hatched specimens were split longitudinally, one half being decalcified by my method (Lorch, 1947*b*) and the other cut undecalcified. It was found that 4 hours' decalcification (4 changes of buffer at 10° C. and pH 4·7) was adequate, but times up to 4 days did not result in an appreciable loss of enzyme. Reactivation was carried out at room temperature for 24 hours.

All specimens were dehydrated in alcohol, cleared in cedarwood oil or benzene, and embedded in paraffin (m.p. 56° C.). Serial sections (8μ) were mounted without albumen. For the visualization of alkaline phosphatase Gomori's (1939) method as modified by Danielli (1946) was mainly employed. Incubation times were varied from 1 to 15 hours at 28° C. Some sections were treated by the diazo-method of Menten, Junge, and Green (1944), by means of which phosphatase can be visualized independently from calcium salts. However, only sites of very great enzyme activity can be demonstrated (Lorch, 1947*a*). The two-colour method involving the use of gallamine blue (Lorch, 1947*b*) was used to demonstrate calcium salts and phosphatase in the same section. Calcium deposits were also visualized by von Kossa's silver nitrate method, by staining the section for 5–15 mins. in a saturated neutral solution of gallamine blue (Stock, 1949), and by bulk alizarin red S staining. The usual histological stains were employed to elucidate general structure. A combination of methyl green, which stains cartilage metachromatically, and van Gieson's stain was found useful.

RESULTS

The description of phosphatase distribution is based on a study of 13 specimens which may conveniently be divided into 6 stages (see opposite). Purely anatomical features will not be described; reference should be made to de Beer (1931, 1937).

Stage	*Length (mm.)*	*No. of specimens*
1	18–20	2
2	32–35	2
3	47	1
4	58	1
5	75	2
6	96–115	5

1. *Cartilage of the Skull*

At the earliest stages examined (18–20 mm.) no cartilage is present. The first signs of precartilaginous tissue appear in the region of the future parachordals as condensation of mesenchymal cells. No phosphatase was detected in this tissue, nor in the developing cartilages of the 32–35-mm. specimens. At Stage 3 (47 mm.) the main features of the adult skull are established. The growing regions and the more recently formed cartilages are still negative for phosphatase, but a positive reaction is now given by the chondrocytes of some older cartilages. Centrally placed cells are only faintly positive but the flattened chondrocytes at the periphery stain strongly. Perichondral fibroblasts and fibres display phosphatase activity in most cases but never at the growing zones. In the parachordals, the oldest cartilages of the skull, the cells stain strongly and traces of phosphatase occur also in the matrix. Meckel's cartilage of the lower jaw may be described as typical of this stage: its anterior tip is just distinguishable from the connective tissue. It has no phosphatase activity. Distally, Meckel's cartilage is better defined and has a distinct positively staining perichondrium on the dorsal and ventral surface. Laterally the cartilage merges into connective tissue. Pl. I, fig. 1, shows the distribution of phosphatase in two jaw cartilages of a 47-mm. specimen. In the 58-mm. embryo the anterior parts of Meckel's cartilage are partly surrounded by positively staining perichondrium, leaving the central (growing) surfaces free. But in the mid-orbital region the strongly staining perichondrium forms a complete ring round the cartilage and patches of strongly positive chondrocytes make their appearance near the ventral edge. The matrix is also slightly positive here. Similar patches are seen in the pterygoquadrate and ceratohyal. Apart from these occasional well-localized patches the cartilage matrix is negative at this stage, but nuclear phosphatase occurs in some of the flattened peripheral chondrocytes and in the perichondral fibroblasts.

At Stage 5 a new phenomenon makes its appearance: the jaw cartilages, parachordals, and most of the brain case of the 75-mm. and older specimens display phosphatase activity in a well-defined pattern. The cartilage may be divided into three zones (Text-fig. 1). Immediately below the perichondrium the 2–3 rows of flattened cells continue to display phosphatase activity and the matrix may give a moderately strong reaction (Zone 1). This region shows less affinity for basic dyes than the rest of the cartilage matrix. Passing inwards the cells become more rounded and their phosphatase activity increases sharply. Pericellular 'capsules' of intensely positive matrix are frequent. The matrix between the cells also stains strongly (Zone 2). Centrally to this layer of cells the cartilage abruptly loses its phosphatase activity, although it does not appear in any way different when ordinary staining procedures are used (Zone 3). The zones are best distinguished after relatively short incubation times and it becomes clear that the maximum phosphatase activity is found in Zone 2. When the growing region of a cartilage is examined it is seen that phosphatase appears in the following order: first in the perichondrium as soon as this is clearly defined, secondly in 'Zone 1' (flattened cells), and finally in the cells and matrix of Zone 2 where the reaction is most intense.

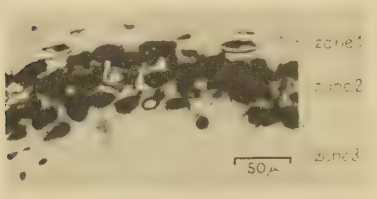


FIG. 1

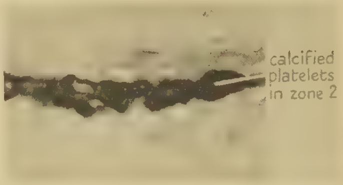


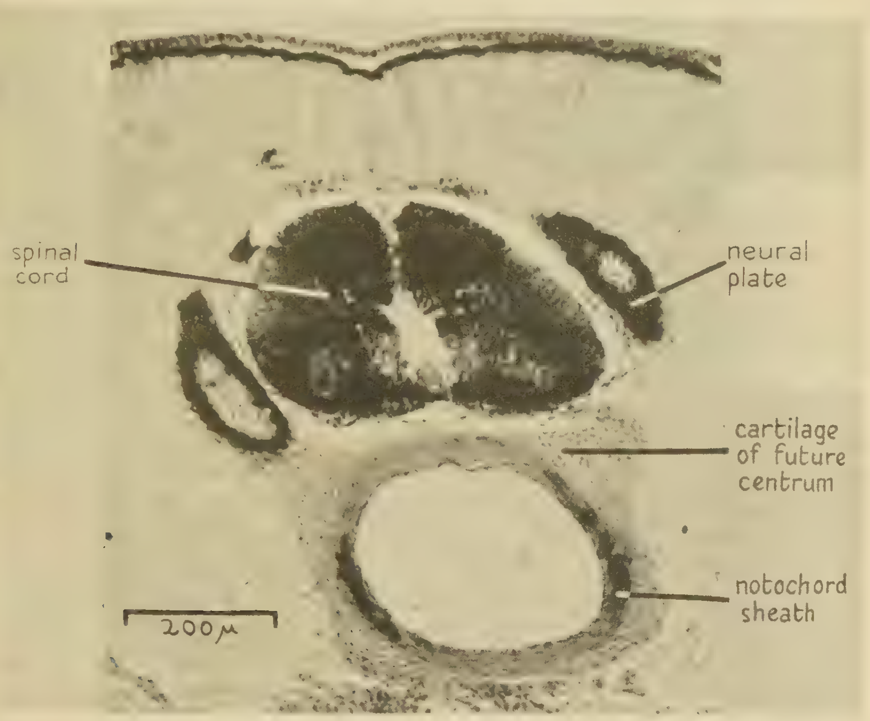
FIG. 2

TEXT-FIG. 1. From a decalcified transverse section through the pterygoquadrate of a 110-mm. specimen, showing the distribution of phosphatase at the periphery of the cartilage. Incubation time, 5 hours.

TEXT-FIG. 2. Calcified platelets at the periphery of the parachordal cartilage of a 115-mm. specimen. Stained with gallamine blue and counterstained with safranin. Note position of platelets corresponds to 'Zone' 2 in Text-fig. 1.

As growth proceeds Zone 2 increases somewhat in thickness, but the negative core of cartilage (Zone 3) persists even in adult dogfish. Pl. I, fig. 2, shows the appearance of Meckel's cartilage and the pterygoquadrate at Stage 5.

In the skulls of embryos up to Stage 6, there was no sign of calcification detectable by histochemical methods. But in the hatched specimens (96–115 mm.) calcified platelets are seen at the periphery of some of the cartilages (Text-fig. 2). With regard to the distribution of phosphatase in these specimens there is no qualitative change in the pattern just described. However, Zone 2 is now present in a greater number of cartilages and has increased in thickness in places where it was previously found. The reaction is even more intense and can be detected after very short incubation times (1 hour). It is of interest to note that the calcified platelets are always located within Zone 2. The phosphatase reaction is more extensive than the region of calcification, but the reverse was never the case. The perichondrium, previously positive, now appears negative. Some of the smallest branchial cartilages are still devoid of phosphatase, whereas others show a patchy distribution of Zone 2. Meckel's cartilage, the pterygoquadrate, and the ceratohyal have broad, intense bands of phosphatase activity, and show most extensive peripheral calcification. In the anterior region of the parachordals extracellular phosphatase is present only near the ventral surface, whereas more distally ventral and dorsal positive bands are seen. Calcification is strictly correlated with this phosphatase distribution. Decalcified sections treated with haematoxylin and eosin or van Gieson's stain do not show any change in the matrix in the region of calcification, such as takes place in the calcifying cartilage of mammals, nor is there any hypertrophy of the phosphatase-containing cells similar to that seen during cartilage-bone formation in the trout (Lorch, 1949). In cartilages of an adult dogfish examined, essentially the same distribution of phosphatase was found as in the calcified cartilages of the recently hatched specimens.



TEXT-FIG. 3. Transverse section through the vertebral column of a 47-mm. specimen. Phosphatase shown black (Gomori method). There is no calcification. Incubation time, 4 hours.

2. *The Vertebral Column*

Only the trunk region will be described. Up to the 35-mm. stage no phosphatase was detected in the notochord or in the rudiments of the vertebral cartilages. However, at Stage 3 (47 mm.) intense, well-localized areas of phosphatase activity are found. The matrix at the periphery of the neural arch cartilages is strongly positive as well as the chondrocytes both at the periphery and, to a lesser degree, in the centre (Text-fig. 3). Only the cartilage at the junction of neural plate and notochord sheath is entirely free from phosphatase. The intercalary tissue and the perichondrium are negative. Zones of phosphatase activity display an affinity for acid dyes: the periphery of the neural arches stains pink with eosin or acid fuchsin and red with Heidenhain's Azan.

The spongy tissue of the notochord displays no phosphatase activity at this and subsequent stages, but the fibrous sheath foreshadows the future double-cone pattern of calcification in the vertebral centra by the distribution of phosphatase in the nuclei of spindle-shaped concentrically arranged cells. There is as yet no calcification in the vertebral column.

At Stage 4 (58 mm.) the pattern of phosphatase distribution becomes more sharply defined and the reaction more intense (Pl. I, fig. 3). The strongest reaction (very marked after 20 minutes' incubation) is given by the cells and fibres of the notochord sheath but only in the area of future calcification. The intervening tissue is negative, although as yet no histological differentiation can be seen within the future centrum. In the neural plates the cells and matrix are now positive throughout the cartilage, but short incubation times (1 hour) and application of the diazo-method show that the most intense reaction occurs in the matrix at the periphery (Text-fig. 4). The first signs of the calcification are now seen in the matrix just below the peripheral layer of flattened cells which is very narrow in the vertebral cartilages (Pl. I, fig. 4). No platelets of calcium salts similar to those in the skull have been noted. Sections treated by von Kossa's silver nitrate method show a granular deposit (Text-fig. 5). It is again noted that the phosphatase reaction is more widespread than the zone of calcification, but deposits of calcium salts do not occur in negative regions.

In the 74-mm. embryo calcification of the neural plates has progressed considerably: only the central core of the cartilage is now free from calcified deposits. There is a tendency for the granules to fuse and form a homogeneous calcified zone (Pl. I, fig. 6). In the specimens above 95 mm. granular deposits are no longer seen. The phosphatase activity of the neural plates becomes less as calcification increases: at Stage 5 some positive chondrocyte nuclei are encountered within the most recently calcified zone, but heavily calcified regions are quite negative (Pl. I, fig. 5). At the junction between the calcified outer layer and the uncalcified core of cartilage the matrix continues to display phosphatase activity. Hence it appears that the phosphatase positive zone is progressively pushed inwards by the advance of calcification. There is some overlap when phosphatase and calcium salts occur in the same region.

The matrix of the calcified cartilage becomes increasingly more acidophil, while uncalcified cartilages such as the neural spines show the usual affinity for basic dyes. In the hatched specimens the strongly calcified periphery of the neural plates have irregularly shaped lacunae which contain the chrondrocytes. The latter also show various shapes and do not resemble the spherical chondrocytes of the non-calcified cartilage which have basiphil granules in the cytoplasm. In the transition zone (phosphatase positive) some chondrocytes have a strongly acidophil cytoplasm devoid of granules, while others are still granular.

The notochord sheath first shows a zone of calcification in the 75-mm. embryo, i.e. somewhat later than the neural plates. The pattern already established in the 47-mm. specimen by the phosphatase distribution is maintained. The calcium deposits occur in the middle zone of the fibrous sheath, where a layer 2–3 cells thick stains heavily with silver nitrate (Pl. I, fig. 6). The elongated cells, surrounded by calcium salts, are still well defined and display some phosphatase activity. But in the oldest specimens, where the calcified zone is much thicker, the cells are negative.

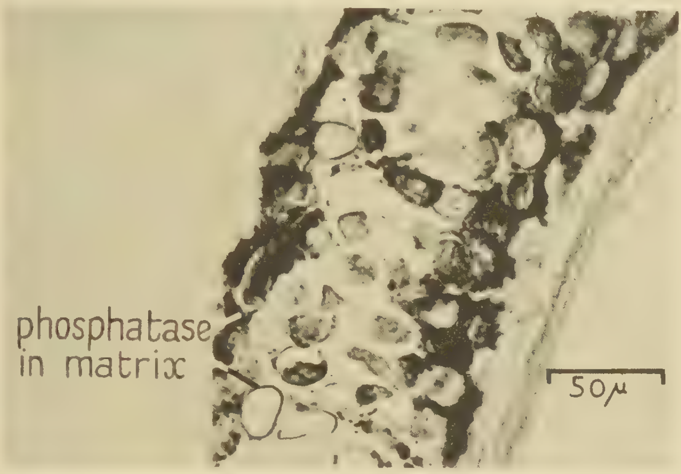


Fig. 4

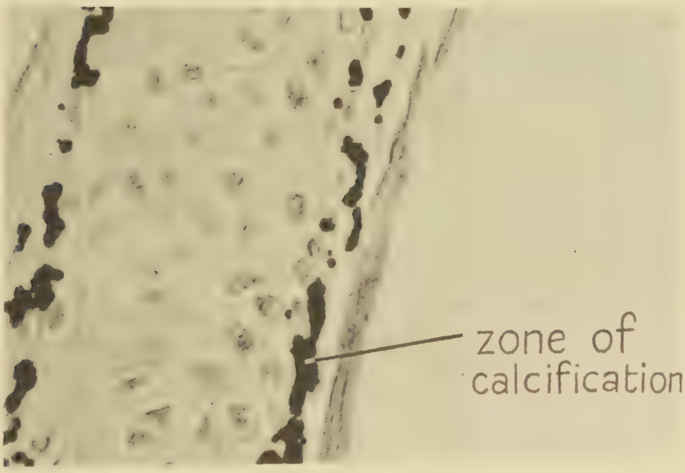


Fig. 5

Text-figs. 4 and 5. From the neural plate of a 58-mm. specimen. In Fig. 4 phosphatase is shown as Ca α naphthyl phosphate (modified method of Menten, Junge, and Green, 1944). Fig. 5 shows the distribution of calcium salts (von Kossa's nitrate method).

The inner zone of the fibrous sheath displays strong phosphatase activity in all 74–115-mm. specimens, but the outer zone becomes progressively less positive. In the intervertebral regions there is no calcification and no phosphatase. No secondary calcification of the outer zone of the notochord sheath was noted. This is said to occur (Hasse, 1893).

The relation between phosphatase and calcification in the skeleton of the series of dogfish embryos examined is summarized in Table 1.

Discussion

The following points emerge clearly from this study: phosphatase is absent from the cartilages of the young embryos and appears first in the chondrocyte nuclei and in the perichondrium. At a stage just prior to calcification the enzyme is also detected in the cartilage matrix. The maximum extracellular phosphatase activity occurs during the first stages of calcification. The phosphatase positive zone then retreats in front of the wave of calcification. This is most clearly seen in the neural plates where calcification has reached the most advanced state. In the skull the stage of secondary reduction of phosphatase activity has only just been reached in the hatched specimens. It seems, therefore, that in dogfish, as in teleosts and mammals, phosphatase is an essential pre-requisite for calcification. Moreover, it is noted that calcification only occurs in regions where *extracellular* phosphatase is present, but there is a lag between the appearance of the enzyme in the cartilage matrix (or notochord sheath) and the onset of detectable calcification. It is interesting to note in this connexion that no extracellular phosphatase could be detected in the cartilage and notochord sheath of an adult lamprey (*Petromyzon fluviatilis*), although the peripheral chondrocytes gave a positive reaction as in young dogfish embryos. The skeleton of cyclostomes does not calcify. To save space no description of the distribution of phosphatase in tissues other than the skeleton has been given, but it must be understood that the enzyme is present in tissues other than those here described. Taking the embryo as a whole the conclusions reached with developing trout (Lorch, 1949) are confirmed: nuclear phosphatase shows no relation to calcification, whereas extracellular phosphatase is only found in connexion with calcification or fibre formation.

There is one notable difference between calcification in the dogfish and cartilage bone formation in the trout: in the latter the chondrocytes enlarge, the matrix becomes basiphil and a perichondral layer of pre-osseous substance is formed prior to calcification. However, in *Scyliorhinus* the calcium salts are precipitated within the cartilage and neither the matrix nor the cells show any preliminary change other than the phosphatase distribution. (The change in staining reaction described by Weidenreich [1930] and noted in the vertebrae of the older specimens is secondary to calcification.)

Whereas one cannot exclude the possibility of phosphatase playing a part in the formation of the pre-osseous matrix in teleosts and mammals, such a role is unlikely in elasmobranchs. The simplest hypothesis is therefore that

TABLE I

STAGE	SKULL CARTILAGES				VERTEBRAL COLUMN				REMARKS
	PHOSPHATASE IN			CALCIFICATION OF MATRIX	NEURAL PLATES		NOTOCHORD SHEATH		
	PERICHONDRIUM	CHONDROCYTES	MATRIX		PHOSPHATASE	CALCIFICATION	PHOSPHATASE	CALCIFICATION	
1									NO CARTILAGE; PRECARTILAGE NEGATIVE.
2	—	—	—	—	—	—	—	—	
3	+	—	—	—	+	—	+	—	
4	+	+	+	—	++	+	++	—	
5	+	++	+++	—	+++	++	+++	+	
6	+	++	+++	+	+	+++	++	++	
ADULT	—	+	++	+++					VERTEBRAE NOT EXAMINED

phosphatase is here solely concerned with the precipitation of calcium phosphate.

ACKNOWLEDGEMENTS

I should like to thank Professor J. F. Danielli for reading the manuscript and Professor Samson Wright for providing facilities for this work, which was financed by a grant from the Medical Research Council.

SUMMARY

1. A histochemical study has been made of alkaline phosphatase in the endoskeleton of dogfish embryos of 18–115 mm.

2. The distribution of phosphatase is compared with that of insoluble calcium salts.

3. Phosphatase was first noted in the chondrocytes and perichondrium at 47 mm. and in the cartilage matrix at 58 mm.

4. Calcification occurred first in the neural plates at 58 mm. and in the skull at 74 mm. No calcification was observed in zones devoid of extracellular phosphatase.

5. As the intensity of calcification increased the amount of phosphatase tended to drop.

6. It is concluded that in elasmobranchs, as in higher animals, extracellular phosphatase is an essential precursor of calcification.

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DESCRIPTION OF PLATE I

FIG. 1. Transverse section through the jaws of a 47-mm. specimen. Sites of phosphatase activity black (Gomori method). Incubation time, 3 hours. There was no calcification.

FIG. 2. Transverse section through the jaws of a 75-mm. specimen. Sites of phosphatase activity black (Gomori method). There was no calcification. Note the patchy distribution of phosphatase in the cartilage matrix. Incubation time, 3 hours.

FIGS. 3 and 4. Serial transverse sections through the vertebral column of a 58-mm. specimen. Fig. 3 incubated 6 hours for phosphatase visualization (Gomori method). Fig. 4, unincubated control showing zone of calcification.

FIGS. 5 and 6. Serial transverse sections through the vertebral column of a 75-mm. specimen. Fig. 5 shows the distribution of phosphatase in a decalcified section (Gomori method, 3 hours' incubation); Fig. 6 shows the extent of calcification (von Kossa's method). Note the regression of phosphatase from heavily calcified zones.

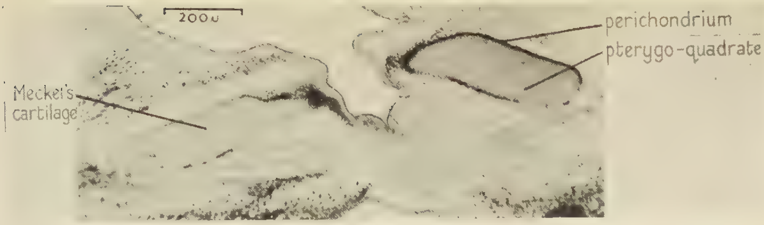

FIG. 1

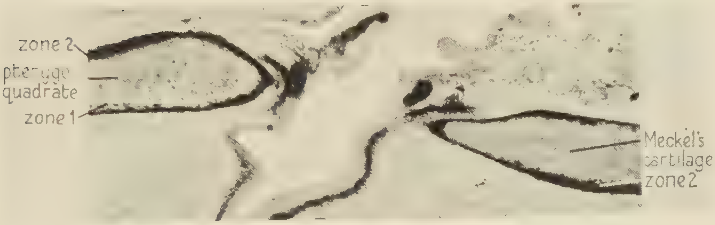

FIG. 2

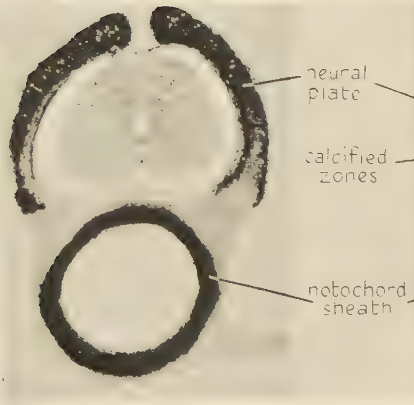

FIG. 3

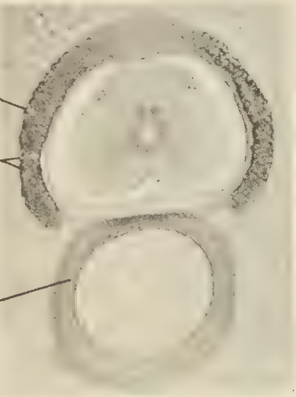

FIG. 4

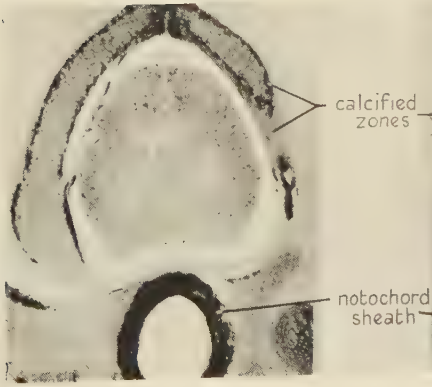

FIG. 5

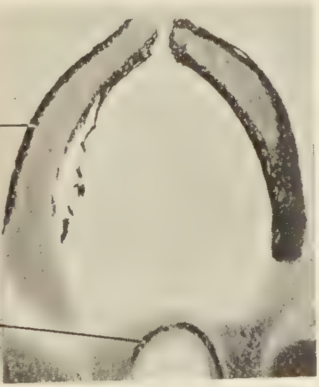

FIG. 6

J. LORCH—PLATE I

Alkaline Phosphatases and the Cycle of Nucleic Acids in the Gonads of Some Isopod Crustaceans

BY

MARINA DE NICOLA

(*From the Centro di Citogenetica del C.N.R. Università di Napoli*)

With one Plate

INTRODUCTION

MUCH work has been done with a view to localizing alkaline phosphatases in various tissues by means of the technique of Takamatsu (1939) and Gomori (1939). The advantage of this technique is that it is based on a chemical reaction and thus highly specific, at least for a certain group of enzymes. Furthermore, critical observations, mainly by Danielli (1946) and Lison (1948), indicate that this technique not only shows great specificity but also makes it possible to localize fairly exactly the sphere of the enzymatic action; one can assume with a reasonable degree of safety that the areas which appear positive exercise *in vivo* enzymatic activity.

Recent work by Krugelis (1947) on proliferating tissues of certain marine invertebrates has shown a varying distribution of alkaline phosphatases in the various stages of development. The author has come to the conclusion that their distribution does not completely coincide with that of the ribonucleic acid in the cytoplasm.

The present work was carried out on the gonads of some Isopod Crustaceans (*Asellus aquaticus*, *Meinertia parallela*, and *Anilocra physodes*) with a view to controlling the latter point and finding out what relation exists between the phosphatases and ribonucleic (RNA) and thymonucleic (DNA) acid. As they are phosphorus compounds it is likely that phosphatases are concerned in their metabolism.

So far, little is known either of the chemical nature of phosphatases or of their classification. All that can be said is that they break the bonds between phosphoric acid and various organic radicles.

Phosphatases have been classified by Lison (1948) into monoesterases and diesterases. Monoesterases act upon compounds of the type $R_1O.PO_3H_2$, whereas diesterases act upon compounds of the type $R_1O(R_2O)PO_2H$. The technique of Takamatsu and Gomori reveals the presence of alkaline phosphatases by splitting off phosphate from glycerophosphate: the phosphate is precipitated by the calcium present in the medium, and the precipitate, which may be visualized by several methods, reveals the site of the enzyme.

In the case of the diesterases it is doubtful whether a precipitable compound would be formed as a result of enzyme action: the action would normally be

$$R_1O(R_2O)PO_2H \rightarrow R_1O.PO_3H_2 + R_2OH,$$

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

and both the compounds $R_1O.PO_3H_2$ and R_2OH will normally be as soluble as, if not more soluble than, the initial compound. But one of the two products, $R_1O.PO_3H_2$, is a substrate for monoesterases: consequently in tissues which are rich in alkaline phosphatase the products of the activity of diphosphatase may be subjected to the action of monoesterase before they have diffused far. Thus, if a tissue rich in monoesterase is exposed to the substrate for diesterase, the site of diesterase may be indicated in a rough way by the product of the consecutive reactions diester→monoester→free phosphate. The localization of diesterase so obtained must necessarily be lacking in precision, and of uneven value.

Here care has been taken to distinguish these two types of enzymes, mono- and diphosphatases. As substrate for monophosphatases sodium-β glycerophosphate, and for diphosphatases RNA and DNA were used.

A preliminary note of the results presented in this paper was published in *Experientia* (Montalenti and de Nicola, 1948*b*).

MATERIAL

The material used was the gonads of *Asellus aq.* and of some Cymothoids (*Anilocra physodes* and *Meinertia parallela*). The stages investigated were: in spermatogenesis, from spermatogonia to spermatocytes, spermatids, and spermatozoids; in oogenesis, from oogonia to oocytes in diplotene and first stage of the formation of auxocytes, when the chromatin is in the so-called 'diffuse stage'.

TECHNIQUE

The technique used was Gomori's method with slight modifications as already suggested by other workers.

The best fixation obtained was with acetone at a temperature of 4° C. for 12–24 hours, followed by treatment with abs. alcohol for 1 hour. Then 10 minutes in xylene, followed by embedding in paraffin (changed twice) at 52° C. for a total length of 15 minutes. The pieces used were very small. They were immediately sectioned at 5μ and the paraffin was removed within 12 hours. They were then put in an incubator at 38° C. Some sections were kept in the refrigerator for a few days and did not lose their enzymatic activity. The incubation solutions were, as recommended by Gomori, at pH 9·5, only the substrates being different. These consisted of sodium-β glycerophosphate for monophosphatases, polymerized DNA (containing 1·2 per cent. protein), and RNA.

The duration of incubation varied, according to the kind of material, from 8 to a maximum of 72 hours, for the nucleic acids. Due consideration was taken of recent objections to Gomori's technique. It is assumed, however, that the technique used, i.e. fixation at a low temperature, rapid embedding, and rapid paraffin removal, should reduce to a minimum the unavoidable partial inactivation of the enzyme during the histological preparation.

Control sections were kept at 90° C. for 15 minutes before incubation, or in $Hg(NO_3)_2$ for an hour at normal temperature. This was observed to produce inactivation of the alkaline phosphatases (Montalenti and de Nicola, 1948).

By means of this technique the cellular structure remains unaltered and phosphatases are entirely destroyed. Furthermore, other control sections were kept in Gomori's solution without substrate for the same length of time. They always remained unstained. In all slides the staining was equally distributed and of constant intensity, which proves that there are no perceptible variations due to technical errors.

OBSERVATIONS

Spermatogenesis and Polyploid Nuclei of Follicular Cells

Experiments were carried out during various stages of the spermatogenesis of *Asellus aquaticus* and Cymothoids. The general result was that the phosphatases decrease during the development from spermatogonium to spermatocyte and to spermatid. They are completely absent in the latter with the exception of some traces along the external membrane and particularly at one point in it. Slightly positive results are obtained in the spermatozoids (Pl. I, fig. 2). This decreasing intensity of staining finds its counterpart in a similar behaviour towards ordinary nuclear stains.

The chromosomes are always positive for phosphatase (Pl. I, figs. 1 and 3). The nuclei of the follicular cells of the testis of *Asellus*, i.e. the giant polyploid nuclei, undergo a cycle of variations which may be related to that of the germ cells (Vitagliano, 1948). They give rise to RNA, which is put out in form of granules which pass between the germ cells. The relationship between the nucleic acids during this process was investigated (de Nicola, 1948) in order to ascertain what part the phosphatases play during the synthesis of RNA. Four stages of polyploid nuclei have been described with ordinary nuclear stains, corresponding to four stages of distribution of phosphatases:

Stage I: Nucleus with granular chromatin. One or two nucleoli present. Small quantity of RNA. In the cytoplasm it is almost completely absent. Monophosphatases and diphosphatases exist in nucleoli and in the chromatin granules, not in the cytoplasm.

Stage II: Nucleus with diffuse chromatin. Five or six nucleoli. Small granules of RNA in the cytoplasm. Monophosphatases only in the nucleoli. Diphosphatases only in two or three nucleoli. It must not be overlooked that RNA is present only in two or three of these nucleoli while all of them stain by iron haematoxylin.

Stage III: Nucleus much contracted and intensely Feulgen positive. Cytoplasm full of RNA. One or two nucleoli. During this stage the polyploid cells produce the maximum quantity of RNA granules. Great quantities of monophosphatases and diphosphatases in the nucleus.

As was pointed out in the publication of Vitagliano (1948), stages II and III occur twice during the spermatogenetic cycle before the onset of the fourth stage.

Stage IV: Nucleus less contracted than in the previous stage, chromatin in form of numerous Feulgen-positive granules. One or two nucleoli. Cytoplasm presents moderate quantity of RNA. Monophosphatases and diphosphatases are present in the granules of the chromatin and in the nucleoli.

In conclusion, the phosphatases exist only in the polyploid nuclei and in the nucleolus of the germ cells: they correspond in these two areas to the distribution of the nucleic acids. They are completely absent in the cytoplasm and the RNA granules which are expelled from the cytoplasm.

Thus phosphatases exist only where there is a synthesis (polyploid nuclei) or re-elaboration (nuclei of germ cells) of the nucleic acids. The cytoplasm, which plays no part in this process, is devoid of these enzymes. The granules, which are no longer in metabolism during their way from polyploid nuclei to germ nuclei, do not contain any phosphatase though they are rich in RNA.

The polyploid nuclei of the follicular cells of the testis of Cymothoids are also strongly positive (Pl. I, fig. 2), but I did not investigate whether stages similar to those mentioned above can be recognized in this material.

OOGENESIS

Monophosphatases

A progressive decrease of enzymes is observed in the course of the development from the oogonium in *Asellus aquaticus*: typical chromosome figures are stained by Gomori's reaction (Pl. I, fig. 3). The cytoplasm becomes considerably less positive during development. The same applies to the nucleus though the reaction is never totally negative.

Identical observations were made with Cymothoids where more detailed observations could be made, owing to the large size of the oocyte. One, or sometimes (especially in earlier stages) two nucleoli are observed which react with the same intensity in all stages (Pl. I, fig. 6). Chromocentres were also noted, consisting only of DNA showing a positive reaction to monophosphatases and diphosphatases of DNA.

In the remaining part of the nucleus phosphatases are distributed according to the chromosome structure typical for each species. The form of the bivalents at the beginning of the 'diffuse stage' shows, indeed, a marked and typical difference between *Meinertia parallela* and *Anilocra physodes*. In the former the bivalents form small blocks of irregular outline while in the latter they retain their elongated and filamentous shape. These structures as they appear by means of ordinary nuclear dyes correspond exactly to the structures obtained by Gomori's reaction (Pl. I, figs. 4 and 5).

In the oocyte at the maximum size observed, in which vitellogenesis is well advanced and the chromatin practically all diffuse, the phosphatase reaction is very weak (Pl. I, fig. 6). The quantity of phosphatases is considerable in the cytoplasm during the earlier stages. It decreases and almost disappears as vitellogenesis proceeds (Pl. I, fig. 6).

The small diploid nuclei of the follicle cells show a very intense reaction for monophosphatases, and DNA and RNA diphosphatases. They do not present any cycle which could be compared with that of the polyploid nuclei of the testis.

Diphosphatases

As mentioned above, experiments were also carried out on alkaline diphosphatases using DNA and RNA as substrate.

The diphosphatases of RNA exist in the chromosome structure of the nucleus and—homogeneous and diffuse—in the cytoplasm of all the stages examined. They correspond to the distribution of RNA which is particularly abundant in the nucleus, as I have ascertained by means of the Unna-Pappenheim reaction.

The diphosphatases of DNA show a distribution corresponding to that of DNA in the nucleus; indeed, the characteristic blocks of chromatin appear in *Meinertia*. They also exist in the nucleoli and in the cytoplasm, but in neither of these do they correspond to the distribution of DNA which is completely absent.

Both diphosphatases exist in the nuclei of follicular cells.

DISCUSSION

Monophosphatases

The localization of alkaline monophosphatases closely follows the distribution of DNA and RNA. In the nucleus the structures blackened by Gomori's reaction are identical with these stained by the common nuclear dyes. The nucleolus is always strongly positive. This is not unexpected, because phosphatases, which are dephosphorylating enzymes, may well be present where any synthesis or degradation of phosphorus compounds such as nucleic acids occurs.

Therefore the cycle of phosphatases might be interpreted as simultaneous with the cycle of nucleic acids. In fact, spermatogonia and oogonia are particularly rich in phosphatases, possibly because an active synthesis of nucleic acids takes place in them during successive mitoses and meioses. When the spermatid stage is reached, the synthesis practically comes to its end, and the Feulgen reaction is pale.

The nuclei of oocytes during yolk formation also have ceased to metabolize DNA. The oocyte cytoplasm during the earlier stages is particularly rich in enzyme. This is probably necessary for RNA synthesis, which, in turn, is required for protein synthesis during the subsequent period of enormous increase in size. At the beginning of yolk formation RNA diminishes and eventually disappears entirely. Caspersson (1936–40), by means of ultraviolet absorption, has observed a diminution of RNA running parallel to the size increase, and this has been confirmed by Brachet (1944). The diminution has been confirmed in the material used in this work by means of the Unna-Pappenheim method. Monophosphatase also diminishes and finally disappears almost entirely.

Such a strict correlation of monophosphatase and nucleic acids only exists where the latter undergo synthesis or degradation. In the follicular cells of the *Asellus* testis, only the nucleus, when the synthesis occurs, is rich in enzyme. The cytoplasm, in which a considerable amount of RNA is stored which is not in active metabolism, is entirely devoid of phosphatases. The same is true for the granules which are expelled from the cell nuclei. In the germ cell nuclei, in which synthesis takes place, phosphatases are again to be found.

It may therefore be concluded that the strict connexion between phosphatases and nucleic acids distribution exists only where metabolism of nucleic acids occurs.

Another conclusion may be reached, which is in disagreement with Krugelis's statement. Krugelis (1942) has noticed in various tissues that DNA diphosphatase can be detected only by means of depolymerized DNA. This would be expected since free phosphoric groups are present only after depolymerization. On the contrary, in the present work, a positive Gomori's reaction has been obtained with highly polymerized DNA. This happens with very long incubation time (48 to 52 hours) while the usual time in this material is only 8 hours. Possible explanations are the following: (*a*) DNA spontaneously depolymerizes during the long incubation time; (*b*) a nuclease is present together with phosphatases; (*c*) diphosphatases have also a nucleasic action.

The first possibility may be excluded because incubation solutions previously kept at 38° for 72 hours still require a further 48 hours' incubation with the slides in order to give the reaction on them. Furthermore, the viscosity of incubation solutions kept at 38° for 48 hours was the same as in controls kept in the ice-box. Thus the degree of polymerization is not altered by temperature or alkalinity.

Diphosphatases

The distribution of diphosphatases is more difficult to explain. Their specificity in respect to the substrate has been demonstrated by Krugelis (1947), and is generally accepted; but this author has found a much more localized distribution than that resulting from the present observations. Krugelis has found DNA diphosphatases exclusively in the nucleus and nucleolus, and RNA phosphatase in cytoplasm and nucleolus only. On the contrary, in our material both diphosphatases showed the same distribution. Both DNA and RNA used were carefully purified, and therefore it is improbable that the results are due to impurities.

The distribution of RNA diphosphatases present in nucleus, nucleolus, and cytoplasm corresponds to RNA localization as verified by Unna-Pappenheim's staining method, but they do not follow exactly the RNA cycle. Unlike monophosphatases, they do not show a perceptible diminution in the various stages.

DNA diphosphatases are present in the nucleus on the chromosome structures. Thus they correspond to DNA localization, but they are found also in the nucleolus and in the cytoplasm, when DNA is entirely absent. These data are admittedly hard to explain. Since the nucleolus is involved in heterochromatin transformation one may think that a transformation of DNA into RNA occurs when the chromosomes enter the 'diffuse stage', and that it contains the specific diphosphatases.

In the cytoplasm DNA diphosphatases remain along the exterior border of the nuclear membrane even when the yolk formation has started. They might play a role in the transformation of DNA into RNA which has to be put out from nucleus into the cytoplasm. In fact, DNA diminution in the nucleus somewhat precedes that of RNA in the cytoplasm: when chromatin has already almost disappeared, the cytoplasm is still slightly positive to RNA reaction. The persistence of diphosphatases in nucleus and cytoplasm might perhaps mean that they represent a storage of enzymes of the cell, being independent within certain limits of the nucleic acid cycles.

As to the interpretation of the distribution of diphosphatases, some objections must be raised. First, it must be recalled that in order to demonstrate the diphosphatases by Gomori's method, the simultaneous presence of monophosphatases is required, which must be present to liberate phosphoric acid, which subsequently precipitates as an insoluble phosphate. Thus the possible existence of diphosphatases only cannot be demonstrated by this method. This means that a negative diphosphatase reaction cannot necessarily be taken as a proof of their absence, but may be due to lack of monophosphatases in the proper place.

Furthermore, since the demonstration of diphosphatases depends on monophosphatase localization, it may happen that the substrate, degradated by diphosphatases, shifts towards regions where monophosphatases are present, if these are located nearby. In this way the final reaction would take place where monophosphatases are present and the method would show the localization of the latter instead of diphosphatases.

Undoubtedly this hypothesis has some value. However, it does not appear to apply to the material which is the subject of this paper, for the following reasons: as already stated, polyploid nuclei of the follicular cells of *Asellus* testis at a given stage have six nucleoli, three of which show the monophosphatase reaction only, and three show mono- and diphosphatase reactions. If the above hypothesis were correct, the same reaction would be expected in all of them by using only diphosphatase substrate, owing to the propinquity of nucleoli. That is to say that the mononucleotides set free by diphosphatases belonging to the three first nucleoli would move on to the other three containing only monophosphatases and produce the reaction there.

This does not happen; therefore the diffusion hypothesis must be discarded, at least in this case.

I am deeply indebted to Prof. G. Montalenti for his constant advice and encouragement. I also want to express my deep gratitude to Prof. J. Danielli for reading the manuscript and for helpful advice and criticism.

The marine material was supplied by the Zoological Station of Naples, to which I express my gratitude. DNA and RNA acids, prepared in the laboratories of Prof. Caspersson, were kindly supplied by Prof. J. Runnström and I take this opportunity of thanking him warmly for his kindness.

SUMMARY

1. The distribution of alkaline phosphatases and their relation to nucleic acids have been investigated by means of Gomori's technique in the gonads of Isopod Crustaceans (*Asellus aquaticus* and some Cymothoids).

2. Alkaline monophosphatases are distributed over the Feulgen-positive structures, in nucleolus, and in the cytoplasm of young oocytes. They disappear from every place, except nucleoli, during yolk formation. The close coincidence of their localization with that of nucleic acids indicates that they are involved in the metabolism of the latter. The cytoplasmic localization is probably connected with RNA distribution only where this is being actively metabolized.

3. RNA diphosphatases have a similar distribution; they are present where the acid is present, but they do not follow so closely its cycle. Contrary to monophosphatases, they do not disappear gradually from the cytoplasm of the oocyte, where RNA does so.

4. DNA diphosphatases are partially independent from DNA, because they are present also in some Feulgen-negative structures (cytoplasm and nucleolus). As the RNA diphosphatases, they do not disappear gradually during vitellogenesis.

5. Diphosphatases show also a depolymerizing action on DNA. It seems more likely that this fact is due to a nucleasic action intrinsic in diphosphatases than to an association of these enzymes with a nuclease.

6. The interpretation of diphosphatases as a storage of phosphorylizing enzymes in the cell is discussed.

7. The possibility that Gomori's reaction of diphosphatases fails to show their exact localization in the cell is considered, and, as far as the present material is concerned, discarded.

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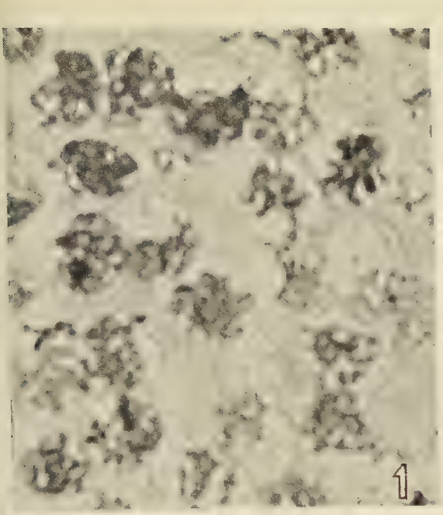
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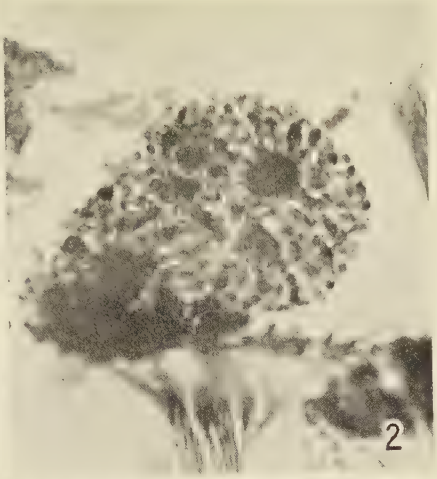
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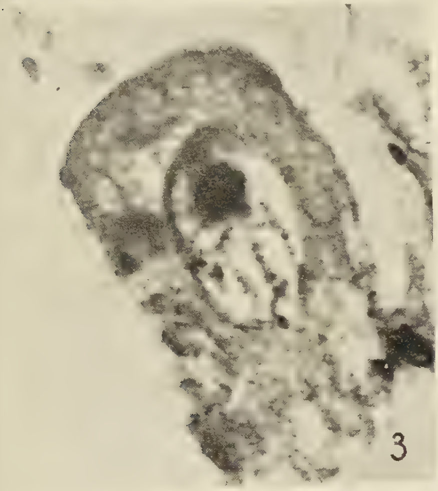
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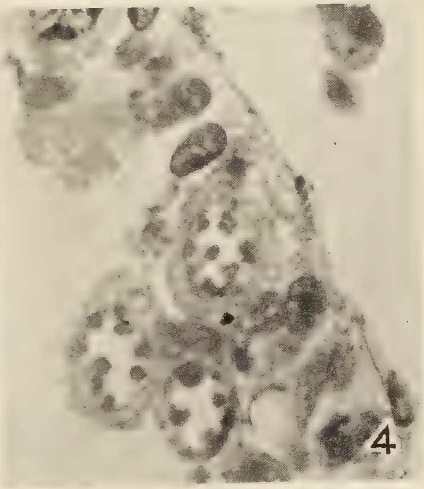
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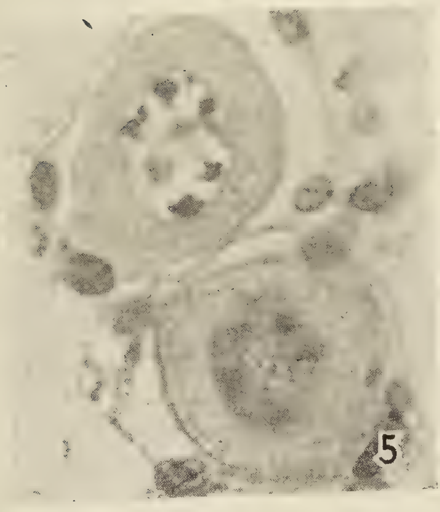
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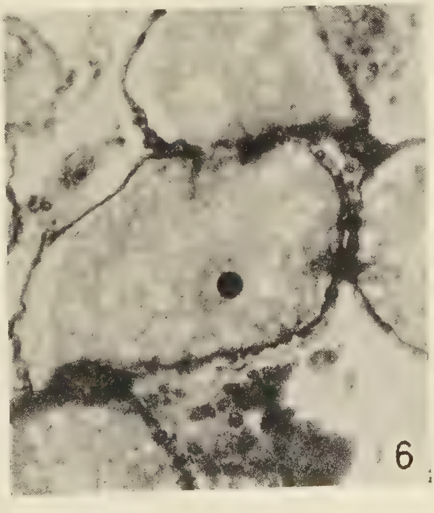
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DE NICOLA—PLATE I

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DESCRIPTION OF PLATE I

FIG. 1. *Meinertia*. Diphosphatases of polymerized DNA: spermatocytes. × 1030.

FIG. 2. *Meinertia*. Polyploid nucleus of follicular cell and spermatozoa. Diphosphatases of polymerized DNA. × 1030.

FIG. 3. *Asellus*. Monophosphatases: oocyte. Chromosomes and nucleolus strongly positive. × 1030.

FIG. 4. *Meinertia*. Monophosphatases: oogonia and young oocytes. The characteristic blocks of chromatin (bivalents) and cytoplasm strongly positive. × 1030.

FIG. 5. *Meinertia*. Monophosphatases: young oocytes. Bivalents and cytoplasm strongly positive. × 1030.

FIG. 6. *Anilocra*. Monophosphatases: oocyte at the beginning of vitellogenesis. Chromatin in 'diffuse stage'. × 285.

Cytochemical Studies on the Embryonic Development of *Drosophila melanogaster*

I. Protein Sulphydryl Groups and Nucleic Acids

BY

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IN experimental embryology the egg of *Drosophila* is known as a type of so-called mosaic egg as established by the experimental work of Geigy (1931*a* and *b*) and others. It is also a well-established fact that mosaic eggs sometimes show differential distribution of particular chemical substances either before fertilization or in very early embryonic stages. Thus the application of cytochemical methods to embryological studies has already yielded results of considerable interest (cf. Needham, 1942; Brachet, 1947*a*). On the other hand, in spite of the fact that *Drosophila* has long been a favourite material for genetic research, comparatively little work has been done on its embryology. It is for these reasons that I considered it advisable to make some cytochemical observations on the *Drosophila* egg. The present paper deals chiefly with the distribution of the protein sulphydryl groups and ribonucleic acid, since these substances are known to have direct bearing on axiation in vertebrate embryos (Brachet, 1947*a*).

MATERIAL AND METHODS

Wild-type Oregon S stock of *Drosophila melanogaster* Meig. was used and cultured by the usual technique at $25 \pm 0{\cdot}2^\circ$ C. Each cytochemical test has been carried out both on developing oocytes and on embryos of different stages. Oocytes in various developmental stages were obtained from 1-day-old females which contain practically all successive stages, whereas some of the intermediate stages are missing if females older than 1 day are used. For the collection of fertilized eggs, virgin females were kept separate from the males for 2 days. They were then mated and the males removed after 1 hour. Thirty to fifty such females were put into a single empty milk bottle and supplied with yeast paste. After another hour or two eggs were collected on a simple medium (2 per cent. agar containing 2 per cent. ethyl alcohol and 1 per cent. acetic acid) on a glass plate. The flies deposited eggs in quick succession under these conditions. After discarding the first two or three lots (which may contain some eggs with advanced embryos), eggs were collected at 10-minute intervals, timed, and incubated. The embryos for study were first de-chorionated free-hand as described by Poulson (1937). It was found that a small piece

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

of the agar medium, after partially drying out, effectively sticks the embryo to the glass plate preparatory to de-chorionation. The embryos were then carefully punctured in the fixative with a very fine tungsten needle. A formal-alcohol-acetic acid mixture (Huettner, 1923) was used as fixative. Gonads and embryos were embedded in the usual way and sectioned at 10μ.

For the detection of protein sulphydryl groups (fixed —SH groups hereafter), the method devised by Chèvremont and Frédéric (1943) was employed. Control sections were treated with saturated mercuric chloride for 1 hour to block the —SH groups. Ribonucleic acid was demonstrated by the method of Brachet (1942): crude ribonuclease was isolated from calf pancreas: sections were incubated in a solution of this enzyme for 1–2 hours at 37·5° C.: control sections were incubated for the same period in distilled water. Desoxyribonucleic acid was studied by the standard Feulgen technique (Stowell, 1945).

In order to get some information about mature sperm, some cytochemical tests were repeated on *Drosophila* testes taken from 1-day-old male flies.

MORPHOLOGICAL OBSERVATIONS ON OOCYTE DEVELOPMENT

Since the development of the *Drosophila* oocyte has not been adequately described, it must first be summarized here. The ovarian history can be divided arbitrarily into eight stages, based on the size of the oocyte and other morphological characters of the nurse cells and follicular epithelial cells. These stages are shown in Text-fig. 1 and a brief description of them is given below (the median cross-sectional area (A) of a drawing of the oocyte was measured by a planimeter under a linear magnification of 160; the diameter (d) of the largest nurse-cell nucleus was measured by an ocular micrometer):

(1) $A = 25$ mm.^{2}, $d = 15\mu$. The oocyte can be distinguished from the nurse cells by its smaller-sized nucleus and less basiphilic cytoplasm.

(2) $A = 160$ mm.^{2}, $d = 20\mu$. The follicle elongates and the follicular cells become flattened out at the nurse-cell end: the nurse-cell cytoplasm becomes strongly basiphilic.

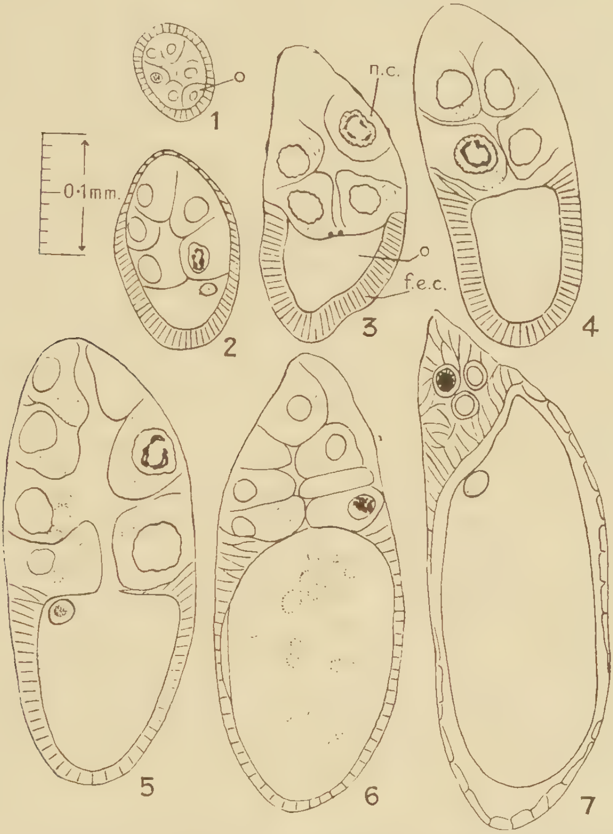
(3) $A = 310$ mm.^{2}, $d = 27\mu$. Deposition of yolk granules starts at the peripheral region of the oocyte. Follicular cells concentrate around the egg: a very thin envelope consisting of a few squamous cells is all that encircles the set of nurse cells (which probably permits an easy access of the raw materials from the body fluid). The cytoplasm of both nurse cells and follicular cells is strongly basiphilic. Intracellular vacuoles appear in the nurse cells.

(4) $A = 560$ mm.^{2}, $d = 40\mu$. Secretory activities of the nurse cells and follicular cells are in full swing. The nurse cells are highly vacuolated and their cytoplasmic contents start to pour into the egg. Not infrequently, whole nurse-cell nuclei can be found inside the ooplasm.

(5) $A = 1{,}080$ mm.^{2}, $d = 40$–42μ. The general features of this stage are similar to those of the preceding stage. However, the physiological activity of the nurse cells has probably passed its peak as evidenced by the decline of

cytoplasmic basiphily: their cell walls are often found broken in the sectioned preparations. The follicular cells show no signs of degeneration.

(6) $A = 2{,}030$ mm.[2] The nurse cells show a steady decrease of cytoplasmic volume and basiphily. Their nuclei became pycnotic, those adjoining the oocyte often being the first affected. A great number of vacuoles can be seen in the oocyte cytoplasm: these are derived from the nurse cells and are observable under living conditions.



TEXT-FIG. 1. Camera lucida sketches of the different stages of the oocyte development of *Drosophila melanogaster*. From iron haematoxylin preparations. *f.e.c.*, follicular epithelial cell; *n.c.*, nurse cell; *o.*, oocyte. Descriptions in the text.

(7) $A = 2{,}600$ mm.[2] The cytoplasmic contents of the fifteen nurse cells are completely absorbed by the oocyte. The follicular cells become flattened, but their cytoplasm is still moderately basiphilic. The whole egg cortex is covered by a thin, homogeneous, yellowish, and refractile structure—the vitelline membrane.

(8) This stage corresponds to a mature oocyte and is not shown in the figure. The follicular cells degenerate and the chorion with filaments is well formed.

No pycnotic nurse-cell nuclei remain visible. The volume of the oocyte increases slightly and a further synthesis of yolk granules occurs.

RESULTS

I. *The fixed —SH Groups*

(*a*) *Oocyte development.* Fixed —SH groups can be demonstrated throughout the ovarian history in both nucleus and cytoplasm of the oocyte, nurse cells, and follicular cells. In the nucleus they are mainly concentrated in the chromatin substances and in the nucleolus. The nuclear sap is negative. In the cytoplasm certain discrete granules always stain more intensely.

In the oocyte the intensity of the —SH reaction increases after stage 3, especially in the cortical ooplasm which stains deep blue as compared with the greenish-blue of the central ooplasm. As maturation proceeds, the distribution of fixed —SH groups becomes more even, but a uniform and higher concentration is still evident in the egg cortex.

In the nurse cells fixed —SH groups are very abundant in the cytoplasm in stages 1–5. Particularly reactive are those areas near the nuclear and vacuolar membranes. Parallel with the decrease of basiphily, the —SH reaction of the nurse-cell cytoplasm weakens in stage 6, whereas the pycnotic nuclei remain strongly positive.

Like the nurse cells, the follicular cells are very rich in fixed —SH groups. In certain stages (3 and 4) an intracellular gradient is evident. The deep-blue granules are accumulated in the proximal half of the cell and concentrated especially near the cell membrane which seems to be connected with the egg cortex by bluish protoplasmic threads. The follicular cells still give a strong —SH reaction in stage 7.

Control sections gave no reaction in most cases. Occasionally the structures containing a high concentration of fixed —SH groups, such as the egg cortex and chromatin materials in nuclei, remained faintly positive after mercuric chloride treatment.

The adult testes are equally rich in fixed —SH groups. Sperm heads are extremely reactive.

(*b*) *Embryonic development.* A cortical concentration of fixed —SH groups is clearly visible in the early cleavage and blastoderm stages which agrees with the distribution already found in mature oocytes. Mitosis is always synchronous in these stages. If the nuclei of an early embryo happened to be in the course of mitosis at the time of fixation, the cytoplasmic —SH reaction was far more intense than that of a corresponding embryo whose nuclei were not in division. This fact is in accordance with the general view concerning the relation between fixed —SH groups and mitosis (cf. Needham, 1942). Like the chromatin substances in a non-dividing nucleus, mitotic chromosomes invariably show a vivid blue reaction.

The posterior pole plasm and later the cytoplasm of the pole cells are particularly rich in fixed —SH groups. However, the pole cells become less reactive after their invagination.

As gastrulation and germ layer formation proceed, the surface ectoderm (or presumptive hypodermis) acquires a greater share of fixed —SH groups. This hypodermal concentration, as a consequence of the cortical localization in the unfertilized egg, is traceable in all parts of the surface ectoderm of the embryo. It is still noticeable at the time of the contraction of the germ band, which takes place at about the 9th hour after laying at 25±0·2° C. It should be pointed out that the temporal course of the morphogenetic events as described here is earlier than those given by Poulson (1937), since the whole embryonic life is shortened by about 3–4 hours at 25±0·2° C. as compared with 22–23° C.

Although the mesoderm, endoderm, and the nervous system rudiment are originally derived from the same lot of blastoderm cells which give rise to the future hypodermis, the former group of tissues contain decidedly less fixed —SH groups than the latter. However, no differences in the intensity of —SH reaction have been noticed between the mesoderm, endoderm, and nervous tissue rudiment themselves.

In later embryos (12 hours or older) an increase of —SH reaction has been observed in certain well-formed organs such as the gut epithelia, salivary gland, gonads, and cuticle. With the exception of the cuticle, the increase of fixed —SH groups seems to be correlated with an increase of cytoplasmic ribonucleic acid.

II. *Ribonucleic Acid*

(*a*) *Oocyte development.* In the stage 1 follicle, nurse cells contain more ribonucleic acid than either the oocyte or follicular cells. In the next three stages (2–4) an enormous increase of cytoplasmic ribonucleic acid occurs in the nurse cells. They now stain deep red with pyronin. The acid content starts to decrease in stage 6.

The follicular epithelial cells give as intense a staining reaction as do the nurse cells in stages 2–5. They retain most of their ribonucleic acid in stages 6 and 7, a fact which is possibly related to the formation of the chorion and filaments of the egg.

At most developmental stages the oocyte cytoplasm takes less pyronin than the nurse cells or follicular cells. The cytoplasm of younger oocytes (before stage 2) often contains relatively more ribonucleic acid than that of older oocytes (stages 3 and 4). In these latter stages the decrease of the acid content is mainly due to a rapid increase in the volume of the developing oocyte. Nevertheless, cytoplasmic materials at the end of the oocyte adjacent to the nurse cells take pyronin as strongly as does the nurse cell's cytoplasm. This indicates the beginning of the nurse-cell absorption. In stages 5–7 the ribonucleic acid content of the egg shows a definite increase as a result of the direct incorporation of all nurse cells, even though the increase in egg volume is more marked than before. The acid content of mature oocytes decreases very sharply again. This is probably connected with further synthesis of yolk materials before maturation. Pasteels (1948) has recently found that in *Ascaris* oogenesis the decrease of cytoplasmic basiphily is correlated with yolk formation. Moreover, Brachet (1942) has mentioned that ribonucleic acid decreases during the oogenesis of *Drosophila.* In the light of the present evidence, Brachet's statement is true only when the comparison is made between certain definite developmental stages.

Ribonucleic acid is not demonstrable in the oocyte nucleus at any developmental stage.

Treatment with ribonuclease completely abolishes the cytoplasmic affinity for pyronin. However, the nucleoli of the follicular cells and corresponding structures of the nurse cells were found to retain some stainability after the enzyme action. Pycnotic nuclei of the nurse cells (in stages 6 and 7) show various shades of colour (green, purple, red, &c.) both in the control and enzyme-treated sections. This probably indicates that depolymerization of desoxyribonucleic acid occurs during the nuclear degeneration, since pyronin will stain this acid only in its depolymerized form (Kurnick, 1947).

The testes from freshly emerged or adult males contain a negligible amount of ribonucleic acid in comparison with the ovaries: only the young spermatogonia located at the anterior end of the testis retain a high ribonucleic acid content. The spermatocytes, spermatids, and mature sperm lose their ribonucleic acid in successive degrees until the mature sperm are practically negative to pyronin. This progressive decrease of basiphily during spermatogenesis is particularly clear when prepupal or pupal testes are studied. A similar phenomenon is known in amphibians (Brachet, 1942).

(*b*) *Embryonic development.* In the newly laid eggs the distribution of ribonucleic acid is diffuse. As soon as the cleavage nuclei begin their migration to the egg periphery, there occurs a simultaneous segregation of ribonucleic acid. Thus, in the incipient or single blastoderm stage, the whole surface blastoderm is so heavily stained by pyronin that it seems as if the ribonucleic acid content is increased. Especially noticeable is the concentration of the acid in the so-called 'innere Blastema' which is later to be partially incorporated with the outer layer of blastoderm cells. No ribonucleic acid is now left in the central yolk except in the thin protoplasmic areas surrounding the yolk nuclei.

Between gastrulation and the contraction of the germ band (3–9 hours embryos) most principal organ rudiments have been laid down. During this period no conspicuous differences in ribonucleic acid content between different germ layers and their derivatives have been observed. Within the ectoderm itself, however, a differential distribution exists—the half of the cell facing the yolk always contains more ribonucleic acid than the other half. Since this condition was found in the ventral, lateral, and dorsal ectoderm as well as in the invaginating stomodaeum and proctodaeum, it is possibly a mere consequence of the concentration of ribonucleic acid in the 'innere Blastema' in the blastoderm stage.

Comparing the degree of basiphily of the embryonic cells of various developmental stages during the first half of embryonic development (0–9 hours), it was found that ribonucleic acid does not show any appreciable decrease during this period, although no persistent increase of basiphily has been observed as in the case of chick embryogenesis (Brachet, 1947*b*).

In older embryos (12–15 hours) a differential increase of ribonucleic acid due to differential acquisition of synthetic activity arises. For example, the mid-gut seems to acquire this ability soon after coiling of the intestine has taken place (between the 11th and 12th hour). Other organs whose ribonucleic acid content is comparable to that of the mid-gut are the proventriculus, gastric caeca, salivary glands, and to a lesser extent the hind-gut. The increase of basiphily in the gut, when it begins to differentiate, has also been reported in the early development of sea-urchin (Brachet, 1947*b*) and of *Limnea* (Raven, 1946) eggs. Organs such as the Malpighian tubes, muscles, fat bodies, and hypodermis contain less ribonucleic acid, though their cytoplasm is still fairly basiphilic. Nerve cells possess the least amount of ribonucleic acid at most stages: the nerve-fibre region is devoid of it.

Between the 15th and 18–19th hours, when the larva hatches, a definite decrease of ribonucleic acid has been observed in most organs. However, the gut epithelia and salivary glands retain their high acid content.

III. *Desoxyribonucleic Acid*

(*a*) *Oocyte development.* Using the Feulgen reaction, Painter and Reindorp (1939) have made a detailed study of nuclear phenomena in the nurse cells of *Drosophila* ovaries. Analysis of the very early follicles in our preparations has confirmed their finding of the general phenomenon of endomitotic growth in the nurse cells. A few points which are relevant to the development of the oocyte as a whole will be mentioned here. In the first place, there seems to be some relation between the physiological activity of the nurse cells and their degree of polyteny. Morphological and cytochemical evidence each indicates that the nurse cells are physiologically most active in the stage 4 follicle. The nuclear diameter of the largest nurse cell at this stage has already reached 40μ which is at least 512-ploid according to Painter and Reindorp. Secondly, the reactivity of the nurse-cell nucleus as a whole towards Schiff's reagent and the number of the Feulgen-positive granules around the nuclear membrane decrease sharply in stage 5. This is then followed by nuclear pycnosis and reassumption of Feulgen positivity. Lastly it may be stated that there is no demonstrable change in the Feulgen reaction of nuclei of either the follicular cells or the oocyte throughout the ovarian history.

(*b*) *Embryonic development.* A rough inspection of the intensisty of Feulgen reaction in developing embryos of various stages indicated that there can be no large-scale synthesis of desoxyribonucleic acid after the stage of the germ-band contraction. This contention was confirmed by an actual study of mitotic frequency. It was found that mitosis occurs most frequently between the 5th and 8th hours after laying at $25\pm0{\cdot}2^\circ$ C. After the 9th hour cell division can be seen in the brain, mesenchyme cells (?), and occasionally in the ventral ganglion; but not in other tissues. This result is in entire agreement with the findings of Poulson (1945).

The Feulgen reaction of advanced embryos (10–18 hours old) does not differ very much from that of the embryos at the time of the germ-band contraction (9 hours). The only difference is found in the nervous system which, by virtue of the small size of the cell nuclei and the continuous reduction in the volume of the organ as a whole, stands out more sharply in the later than in the early embryos.

Since the growth of the larval tissues in *Drosophila* takes place almost exclusively by the process of endomitosis (Cooper, 1938), it would be interesting to know whether endomitotic growth starts during embryonic development. The nuclear size of the cells of mid-gut and salivary glands has thus been compared between the stages immediately after the contraction of the germ band and just before hatching. Although the nuclear size in the latter stage is definitely larger than that in the former, the total increase in nuclear volume amounts to less than 50 per cent. (from about $28\mu^3$ to $40\mu^3$ for the salivary gland cell nuclei). If the relation between the nuclear division cycle and doubling of nuclear volume holds true, this would suggest that endomitosis does not occur during embryonic life.

DISCUSSION

The importance of fixed —SH groups and ribonucleic acid in the determination of the embryo axis in vertebrate development has been particularly emphasized by Brachet (1947*a*). From the present study there is no reason to suppose that such substances also play a prominent role in *Drosophila* embryogenesis. Some concentration of fixed —SH groups and a differential distribution of ribonucleic acid have been found in the ectoderm of early *Drosophila* embryos. However, owing to the general occurrence of these properties, it is difficult to attach any morphogenetic implications to them. Raven (1946) found a similar situation in *Limnaea*.

Cytochemical evidence indicates that there is no detectable decrease of basiphily during the first half of embryonic development. This maintenance of a relatively strong basiphily must be due to some synthesis of ribonucleic acid, for otherwise one would expect a gradual decrease of basiphily parallel to the increase in cell number and the scale of organization of the embryo, as in the case of the sea-urchin (Brachet, 1947*a*, *b*). Nevertheless, since a small change of ribonucleic acid content (giving a small decrease in staining intensity) might have escaped notice, the possibility of the conversion of some ribonucleic acid into desoxyribonucleic acid is not excluded by the present observation. In fact, Brachet (1947*c*) has recently cited some unpublished data that such conversion does occur in some insects. The apparent resemblance between the nucleic acid metabolism of the *Drosophila* embryo and that of the chick embryo must, therefore, await direct confirmation or possibly even disproof.

As histo-differentiation goes on, there is generally a gradual decrease of ribonucleic acid. This is particularly clear in *Drosophila* embryos older than 15 hours (at 25±0·2° C). In certain organs, such as the salivary glands and gut epithelia, however, an accumulation of ribonucleic acid occurs in the course of differentiation. This is possibly related in some way to the normal physiological function of these organs and may well be the result of histo-differentiation.

The very fact that mitosis is of rare occurrence after the contraction of the germ band and the lack of evidence for endomitotic growth during the latter half of *Drosophila* embryogenesis leads me to suggest that the stage of 'germ-band contraction' marks the transition from a growth phase of embryonic development into a differentiation phase. The term differentiation used here refers to visible histo-differentiation rather than to invisible chemo-differentiation. The latter takes place well before 'germ-band contraction', as indicated by Geigy's (1931*a*, *b*) work.

Summary

In the egg of *Drosophila* the distribution of sulphydryl and ribonucleic acid compounds has no apparent connexion with the dorso-ventral organization, contrary to the situation found in the amphibian egg.

Histochemical evidence suggests that the nucleic acid metabolism of the *Drosophila* egg may be similar to that of the chick embryo.

During embryogenesis the contraction of the germ band can be considered as an important morphogenetic stage which marks the beginning of the histo-differentiation of all larval structures.

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A Critique of the Plasmal Reaction, with Remarks on Recently Proposed Techniques

BY

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INTRODUCTION

THERE has been much disagreement between histochemists and biochemists over the chemical basis of the plasmal reaction and the techniques to be employed in demonstrating it. The purpose of this paper is to provide a critique of the methods that have been proposed.

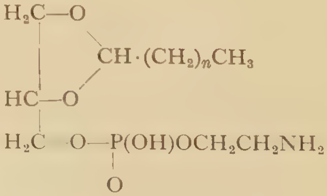
INVESTIGATIONS BY THE FEULGEN SCHOOL

The occurrence in cytoplasm of substances giving a positive result with Schiff's reagent ('fuchsine-sulphurous acid') was first announced by Feulgen and Rossenbeck (1924, p. 230) in a footnote to a paper on the nucleal reaction. In the same year Feulgen and Voit showed that the substances responsible, which were adsorbed on to certain elastic-tissue elements so strongly as to resist paraffin embedding, were aldehydes, as the reaction could be abolished rapidly by phenylhydrazine and by sodium bisulphite. Similar substances found in frozen sections of fresh tissue were demonstrated to be lipoid by their solubility in alcohol; and Voss, who studied the resistant substances (1927, 1928, 1931*a* and *b*), showed (1927, p. 586) that the longer the tissues were in alcohol, the weaker the reaction became.

Feulgen and Voit tried the effect of mercuric chloride on tissues and discovered that the reaction became intensified, or appeared in tissues previously negative; but the same final result could be obtained by the prolonged action of Schiff's reagent, even after treatment with phenylhydrazine, the phenylhydrazone being decomposed by the acidity of the reagent. In tissues negative without the action of mercuric chloride, a preliminary treatment with alcohol abolished the positive reaction after mercuric chloride. Therefore there was a lipoidal precursor which gave rise to the aldehydes, rapidly under the influence of mercuric chloride, slowly in acid media. They named the aldehydes 'plasmal' to mark the distinction in topography between this and the 'nucleal' reaction; the precursor they named 'plasmalogen'. Extracts of tissues were made, the reaction was obtained *in vitro*, and the thiosemicarbazone silver salt of the aldehydes was obtained in the pure state.

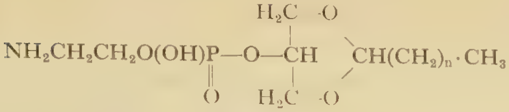
An important series of papers followed, on the occurrence of plasmalogen and plasmal in the body fluids, on their ingestion with food (Voit, 1925; Stepp, Feulgen, and Voit, 1927; Feulgen, Imhäuser, and Westhues, 1928; Imhäuser, 1928), and on their quantitative estimation in body fluids (Feulgen and Imhäuser, 1927; Feulgen and Grünberg, 1939), but these are not specially significant from the point of view of histochemistry. But Imhäuser in 1927 carried out a survey of the occurrence of plasmalogen and plasmal in tissues, and concluded that they were very widespread indeed, in fact that all animal cells contained them, and therefore they must be of fundamental importance in the physiology of the cell. The literature up to 1929 was reviewed by Grevenstuk (1929).

The chemistry of plasmal and plasmalogen was further investigated with great success. Palmital and stearal were investigated by Feulgen and Behrens (1928), who with Imhäuser showed (1929) that plasmal could be isolated and identified by comparison as palmital with an admixture of some stearal. But one other aldehyde at least was present which could easily be detected by its strong smell. This aldehyde could not be isolated, and they believed it to be unsaturated. They had discovered that plasmalogen was always found in the phospholipine fraction on extraction, but it was not itself a phospholipine, or formed from one in the course of extraction, since the content varied according to the source of the extract. Egg yolk, for example, has a very high content of phospholipine and only traces of plasmalogen, whereas the phospholipines of muscle or brain might contain 10 per cent. plasmalogen or more. Separation of the plasmalogen from the phospholipines could be accomplished only by saponifying the latter in alkaline solution; in acid solution, of course, plasmal was rapidly produced. Behrens (1930) prepared plasmal by an improved method, and Feulgen and Behrens (1938) obtained palmitic and stearic acids from it by oxidation. The work was surveyed by Feulgen and Bersin (1939) who showed in addition that plasmalogen could be isolated, though with great difficulty, and that it had the structure of an acetalphosphatide (α or β), that is,



(an α-acetalphosphatide)

or



$$NH_2CH_2CH_2O(OH)\underset{\| \atop O}{P}—O—\overset{H_2C-O}{\underset{H_2C-O}{CH}}\;\rangle CH(CH_2)_n\cdot CH_3$$

(a β-acetalphosphatide)

where the fatty acid radicle is palmityl or stearyl (or the unidentified unsaturated acid). It was observed that the base found was always cholamine. They suggested that choline might replace it, but possibly the acetalphosphatides containing choline were decomposed during the alkaline saponification of the other phospholipines, without which, unfortunately, isolation could not be accomplished. They pointed out also that almost certainly the reason why the acetalphosphatides remained unknown for so long, while the other phospholipines were carefully investigated, was that in the analysis of the phospholipines acid media were invariably used at some stage, and these would destroy the acetal linkages, leaving merely the aldehydes and glycerophosphoric esters. Finally Bersin *et al.* (1941) achieved the synthesis of acetalphosphatide. This appears, unfortunately, to be the last paper published by Feulgen's school; if so, it fittingly terminates their excellent researches.

In the investigation of the occurrence of plasmal, Feulgen and Voit (1924) and Imhäuser (1927) used fresh, unfixed sections, keeping some as controls and treating others with 1 per cent. mercuric chloride. They stuck sections on to slides with albumen, let them dry, and 'fixed' them by quick passage through a flame. They laid very great stress on the necessity for a control section untreated with mercuric chloride—a point that was to be almost completely neglected by other workers on plasmal until Gérard (1935) showed that formaldehyde could be used as a fixative provided it was carefully washed out. This was confirmed by Lison (1936*b*) and the practice was followed by Verne (1947*a* and *b*) but not Verne and Verne-Soubiran (1942). Voss (1927–31*b*) and Verne in earlier and some later papers had used a fixative containing mercuric chloride in order to obtain good fixation, thereby depriving themselves of the control section.

INVESTIGATIONS BY VERNE

The results obtained by the histochemists differ somewhat from those obtained by the Feulgen school, and are not wholly self-consistent. The principal worker on the plasmal reaction has been Verne, who has made a very extended survey of many tissues in different animals, both normal and in various induced pathological states, and has come to extremely important conclusions. He showed (1928*a*) that the plasmal reaction is by no means as widespread as Feulgen and Voit, Imhäuser, and others had thought. Many cells and tissues are completely negative, others (e.g. intestinal epithelium, pancreas, muscle) give only a feeble reaction, but the adrenal, myelin, certain parts of the kidney, and certain cells in the lung are intensely positive. The medulla of the adrenal is weakly positive, the z. glomerulosa, outer part of the z. fasciculata, and inner part of the z. reticularis are strongly positive.

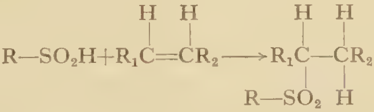
The 'spongiocytes' (fat-laden cells) of the z. fasciculata are very weak or negative, as is adipose tissue. He noted that there was a relation between the reaction and lipoids, but no complete parallel. The reaction with the myelin sheath was so intense that he proposed it as a method for colouring the sheath (1928*b*, *d*) after fixing in a mercuric chloride or platinum chloride fixative and cutting frozen sections. On further investigation of plasmal (1928*c*), in addition to noting that sodium bisulphite and phenylhydrazine block the reaction, and that, as mentioned above, platinum chloride can also be used to provoke its appearance, he distinguished three types of lipoid inclusion: (*a*) positive with sudan III and negative with plasmal, (*b*) positive with both, and (*c*) negative with sudan III but positive with plasmal—the lipoid nature of the last being demonstrated by their solubilities. He made the important observation that by the use of oxidizers, such as potassium permanganate, chromic oxide, or hydrogen peroxide, plasmal-positive bodies could be changed to negative, and plasmal-negative ones to positive, and concluded (p. 268) that such bodies were formed from either neutral fats or phospholipines by oxidation of a suitable hydroxyl group to an aldehyde. He assumes, therefore, that since he has been able to produce positive results by the action of oxidizers on lipoids, those lipoids which already give positive results must already be oxidized in the body and in the same way.

Verne summarized and extended his results in his next paper (1929*a*) in which he makes the interesting remark (p. 248) that fixation should be for at least 6 hours. He points out that, as Feulgen and his collaborators had stressed, a positive result is indicated by a red colour which is much more bluish than that given by Schiff's reagent which has been allowed to become recoloured through the loss of sulphur dioxide. He notes that sebaceous glands are negative, that the interstitial cells of the testis are positive, and that the adrenal is the only other endocrine gland studied which was positive. His results are based on studies of man, dog, cat, cow, calf, sheep, chick, common frog, tench, and eel. In experiments with oxidizing agents he found that adipose tissue can be made positive with plasmal, and that in general, as a lipoid body becomes positive through oxidation the intensity of colouring which it will assume on treatment with sudan III progressively decreases and becomes negative. Sections of adrenal left in water with a trace of mercuric chloride showed a positive result in the z. fasciculata; the same effect was produced in a quarter of an hour by 0·5 per cent. permanganate, or after several hours with 1 per cent. chromium trioxide. He mentions certain renal tubes and pulmonary cells and the adrenal medulla as negative to sudan III and positive to plasmal, and remarks that the pulmonary cells will withstand paraffin embedding. Researches on pure substances showed that pure glycerides and fatty acids (he does not say which), and cholesterol are always negative. Oleic acid and triolein are negative when pure, but become positive after exposure to the air. Unsaturation appears to be necessary, except that saturated aldehydes will give the reaction; unsaturated aldehydes are not present since they cannot be regenerated from the sodium bisulphite compound by dilute acid, whereas the reaction can be made to reappear in tissues after treatment with sodium bisulphite. Consequently he revises his previous conclusion that the aldehydes concerned are formed from alcohols, and now considers that they are formed by oxidation at double bonds, which would explain why it is only the saturated aldehydes that are in question. As aldehydes are notoriously reactive, they are probably in some sort of labile combination in the tissues (those, that is, that have already been produced *in vivo*). The combination is broken by the action of the mercuric chloride or platinum chloride in the fixative. Free aldehydes, he states, have never been found in the tissues. He tried the action of mercuric chloride on various lipoids *in vitro* but could obtain no result.

Verne has not departed from these conclusions, but has strengthened his evidence for them in a long series of papers: on the adrenals (1929*b*), on the action of carotenoids in protecting lipoids from oxidation (1936*a*, *c*), on the acceleration of oxidation by glutathione and colchicine (Verne and Verne-Soubiran, 1939), and especially on the lipoids of the kidney (1937*a*, *c*, 1940).

Investigations by Lison and Gérard

However, certain objections to the specificity of the plasmal reaction had been raised. Lison (1932), after a very careful study of Schiff's reagent, showed that it was by no means specific for aldehydes, although aldehydes were among the most familiar compounds that would give a positive result. He reviewed the work of Wieland and Scheuing (1920) who had finally established the nature of the reaction involved with aldehydes, and concluded that the reaction took place with double bonds in other substances besides aldehydes. It is an addition reaction of the type:



$$\text{R—SO}_2\text{H} + \text{R}_1\overset{\text{H}}{\text{C}}=\overset{\text{H}}{\text{C}}\text{R}_2 \longrightarrow \text{R}_1\underset{\text{R—SO}_2}{\overset{\text{H}}{\text{C}}}—\underset{\text{H}}{\overset{\text{H}}{\text{C}}}\text{R}_2$$

He disagrees with the Feulgen school (Feulgen, Imhäuser, and Behrens, 1929), who claimed that the reaction is a pseudo-reaction with acetone; and he states, in contradiction of Verne (1929*a*), that oleic acid does give a positive reaction, though only after 15–45 minutes. He makes two very important comments: firstly, substances which are not aldehydes and yet give a true reaction will also react with sodium bisulphite, phenylhydrazine, and other reagents supposed to identify aldehydes, and secondly, that the reaction of any such substance with one of these was no guarantee that it would react with others. Consequently, if an unidentified substance in tissues reacts both with Schiff's reagent and with phenylhydrazine, it is not characterized thereby as an aldehyde, nor does it follow that it must react with, say, semicarbazide. This paper is of the highest importance in the interpretation of results obtained with Schiff's reagent.

Lison also remarked that Schiff's reagent could be recolorized by oxidant enzymes if oxygen were present. He was not sure of the interpretation of the colours produced, and thought that the process was probably a pseudo-reaction. Gérard (1935) studied the distribution of plasmal and of 'oxidases' (apparently peroxidases and phenolases according to Lison, 1936*b*, Chapter VI). He used dimedone (dimethyl dihydroresorcinol) to block all aldehyde radicles but found that it did not block the plasmal reaction. Further, there was a complete parallel between the plasmal reaction and the reactions for 'oxidases' except in myelin, and he found that Schiff's reagent was readily recolorized by iodates, periodates, and other oxidizers. As phenylhydrazine acetate does block plasmal, and as the Nadi reaction was positive even after treatment with cyanide (which would seem to rule out an oxidant enzyme) he inferred that there was an oxidizing agent in the lipoids; this was confirmed by the observations that oxidized oleic acid would liberate iodine from slightly acidified potassium iodide (as would sections) and would recolorize some of the reagents for 'oxidases'. Myelin explicitly excepted, his conclusion is (p. 278) that the plasmal reaction when given by inclusions shows a secondarily acquired oxidizing system.

Lison (1936*a*) also studied the Nadi reaction, and noticed that some fats give a feeble positive result with it, which he considered to be due to a direct reaction as it was not inhibited by cyanide, and was improved on boiling. He confirmed Gérard's findings, and added that the substance concerned would give a positive result with benzidine and a peroxidase, and so must be a peroxide. It is formed by atmospheric oxidation at a double bond.

Verne had shown (1937*a*) that in the kidney of the dog there were lipoid bodies that were positive with plasmal, but negative with Nadi, and that tissues which gave a coloration with Schiff's reagent when 'fresh' and after formaldehyde-fixation and treatment with mercuric chloride, did not do so if mercuric chloride were not used. Formaldehyde, in fact, would block the recoloration. He answered the objections raised by Gérard and by Lison, in two papers (1937*b*, 1940) repeating the results just mentioned, and adding that osmium tetroxide, and iridium, platinum, and gold chlorides could be used after formaldehyde-fixation as well as mercuric chloride. (Oster and Schlossman (1942) appear to have discovered the use of gold chloride independently.) His conclusion was (1937*b*, p. 276) that the Nadi reaction demonstrated the progress of autoxidation of the lipoids, whereas the plasmal reaction showed the appearance of products resulting from this autoxidation, which took place at double bonds. Consequently, the lipoids showing only the plasmal reaction ('Feulgen–Verne reaction') must be saturated, all the double bonds being destroyed. Finally he remarks that aldehydes have been detected in rancid oils.

In his second paper (1940) he showed that the plasmal reaction was inhibited by carbon monoxide and cyanide but not by ethyl-urethane. Sections of adrenal and kidney in contact with ethyl-urethane for 24 hours gave a positive reaction as if they had been in water. The reaction could be regenerated after cyanide by mercuric chloride. He thought, therefore, that the oxidation must be mediated by a polyphenol-oxidase. As Lison had shown that the Nadi reaction was unaffected by cyanide, this strengthened Verne's views on the separate natures of the two reactions. Further, the plasmal reaction was blocked by dimedone and could not be regenerated by mercuric chloride. This result was in complete contradiction of Gérard, and Verne considered it as disposing of Gérard's objections. He also claimed that he had obtained on at least one occasion a reduction of ammoniacal silver nitrate by lipoid inclusions, which he took to demonstrate with certainty the presence of aldehyde groups.

Methods and Interpretations

The results of the histochemists are indeed far from presenting the same simplicity, clarity, and coherence as those of the biochemists; nor do they lead to the same conclusions. Also, the technique employed has been varied considerably. There appear to be several distinct reactions, which may or may not have the same basis. The reader may find it convenient at this stage to turn to the table on p. 419. The techniques have been summarized by Cain (1949).

The plasmal technique (that of Feulgen and his colleagues) appears unexceptionable, but unfortunately its users attached sections to slides with albumen by allowing them to dry, and then 'fixing' by passing through a flame. In view of Verne's conclusions on the importance of oxidation at double bonds, it might be objected that the very wide distribution of plasmal as found by the Feulgen school is due at least in part to their method of attaching their sections, provided that the oxidation, once started, is catalysed by mercuric chloride, and by acidity. Also there is the possibility of interference by oxidizers of various sorts as shown by Gérard, and by Lison (1936*a*), provided they are not destroyed by the heating.

The Feulgen–Verne technique has the great disadvantage that there is no control section. Consequently it cannot distinguish between those aldehydes or oxidizers (if any) already present in the tissues and those revealed after the action of mercuric chloride. Yet plasmal is described by its discoverer as being liberated under the influence of mercuric chloride from acetalphosphatide. It follows that the F–V technique cannot be called a 'plasmal' technique.

The method of Gérard would appear to be the best were it not for Verne's very disturbing observation that formaldehyde can block a reaction, present in 'fresh' tissue, which can be revealed again by mercuric chloride. It appears that when discussing this (1937*a*, p. 4) he is referring to a recoloration of Schiff's reagent by fresh tissue without the intervention of mercuric chloride. The effect would be to produce a pseudo-plasmal reaction, because if the tissue were really positive when fresh and there was no intensification of the reaction after treating it with mercuric chloride, then the presence of plasmalogen in Feulgen's sense could not be demonstrated for certain; yet it would appear to be present after fixation. However, the 'plasmal reaction' described by Liang (1947) as being produced by 'fresh' myelin occurs only after several hours' soaking in Schiff's reagent.

The interpretations placed on the different techniques vary correspondingly. The difficulty in evaluating them lies in the fact that lipoid droplets in tissues are never pure and may give rise to a variety of reactions, few of which are really connected. The best evidence is obtained when a reaction is prevented by preliminary treatment with a certain reagent, but there is no guarantee that the reagent is not reacting with other radicles or components at the same time.

Feulgen's interpretation is simple. Acetalphosphatides are decomposed, extremely rapidly with mercuric chloride, to give palmital, stearal, and other higher aldehydes, which give a positive result. Acetaldehyde, studied in urine by Stepp and Feulgen (1921, 1922), occurs in too small quantities to give positive results. It should be noted here that many authors speak of a positive result as the recolouring of Schiff's reagent. This is perfectly true in one sense –the reagent is bleached as it is prepared and now is coloured again—but very misleading in another—the colour is not necessarily or usually the same as that of the reagent recolorized on evaporation of sulphur dioxide, that is, of basic fuchsine. However, too great a reliance on the difference in shade is inadvisable. Lison (1932) showed that very various shades were given in quite genuine reactions; certain authors insist that a positive result is always much bluer than a recolouring produced merely by evaporation; but this is not always true, and in addition, the colour can vary according to the mode of viewing. A section which appears magenta when looked at under the microscope by transmitted light, may appear deep violet when lying on the bench.

Feulgen and his school were able to produce the plasmal reaction with mercuric chloride *in vitro*; and they remark frequently (e.g. Feulgen and Bersin, 1939) that the speed of reaction with mercuric chloride is very high—*fast augenblicklich*. Yet nearly always the reactions described by the histochemists require many minutes to develop, even in the presence of mercuric chloride. Feulgen and Bersin (1939) note that the speed depends to a great extent on the degree of dispersion of plasmalogen; but one would expect to find it in a state of very fine dispersion in the tissues, especially since phospholipines are extremely good dispersing agents. The comparative slowness of the reaction in tissues is very suspicious, particularly in combination with Verne's remark that it is necessary to fix for at least six hours; and the possibility of atmospheric oxidation increases with the time spent in manipulating the tissue. A reaction *fast augenblicklich* has not been described by anyone but Feulgen and his collaborators. One wonders whether a genuine and unequivocal plasmal reaction has ever been seen in fixed tissues.

Verne's great contribution has been to show that a positive reaction with Schiff can be provoked by means of oxidizing agents, and prevented by their continued action. Oleic acid, if exposed to the air, will become positive, as will triolein. And Gérard and Lison have shown that it will then recolorize other leucobases, and that similar substances in tissues definitely appear to form peroxides. Verne maintains, however, that aldehydes are in question. All three agree that the reacting radicle—whichever it is –arises from the double bonds of unsaturated substances. One point requires explanation here. Verne, basing his statements upon the researches of Kaufmann and Lehmann (1926*a*, *b*), remarks (1937*a*, *b*) that osmium tetroxide and sudan III colour only unsaturated lipoids. This is only partly true. Osmium tetroxide will not colour directly many compounds (such as cholesterol and lecithin) which contain double bonds, although it may do so after subsequent treatment with alcohol. Sudan III and the other sudan lipoid-colorants will (as remarked above) colour all lipoids, saturated or unsaturated, provided only that they are neither combined with proteins in such a way as to mask their lipoid nature, nor are they solid—a point which is of great importance here.

It can be shown very easily that sudan black will dissolve in tristearin (for example) *if the tristearin is liquid.* But as the lipoids that are liquids or greases at room temperature, and occur at all commonly in tissues, are invariably unsaturated, Verne is right in practice although theoretically wrong. The reason why he observed lipoids becoming sudan-negative on oxidation would appear to be that the melting-point is being raised by an increase in their degree of saturation.

The various theories may be summarized as follows, the positively reacting substance being italicized:

A. *Feulgen*

$$\text{Acetalphosphatide} \xrightarrow{\text{mercuric chloride}} \text{glycerophosphoric esters} + \textit{aldehydes}$$

B. *Verne*

(i) With mercuric chloride fixation

$$\text{Unsaturated linkages} \xrightarrow{\text{oxidizing enzyme}} \textit{aldehydes}$$

(ii) With formaldehyde fixation

(*a*) $$(\text{Unsaturated linkages} \xrightarrow[\text{enzyme}]{\text{oxidizing}} \text{aldehydes})$$

(*b*) $$\text{Aldehydes} + \text{formaldehyde} \longrightarrow \text{Labile complex}$$

(*c*) $$\text{Labile complex} \xrightarrow{\text{mercuric chloride}} \textit{aldehydes}\ (+\textit{formaldehyde}\ \text{presumably})$$

C. *Gérard* (myelin excepted)

$$\text{Unsaturated linkages} + \text{atmospheric } O_2 \longrightarrow \textit{oxidizer}\ (\text{a peroxide according to Lison}).$$

Feulgen's theory has the support of his very impressive biochemical researches on plasmal and plasmalogen, culminating in their preparation in the pure state, and synthesis. From the emphasis he places on the necessity for a control section it would appear that he obtained positive results with fresh tissues, but did not regard them as necessarily showing plasmal; in this he was right, because what is specific in his technique is the *rapid appearance* of positive substances after the action of mercuric chloride (or of acid solutions for a much longer period). Since a mere recoloration of Schiff's reagent is not specific for aldehydes, nothing can be said about a positive result in fresh, untreated tissue.

Verne suggested (1937*b*, p. 275) that in some cases in which the reaction is negative with fresh tissues the fixative might break down a lipoprotein complex. Where the fresh tissue is positive, he considered that aldehydes are concerned, and that these had been formed already in the tissues from unsaturated linkages, by the same method as he had used to produce a positive result after fixation, that is, by oxidation. The production of positive results with the Nadi reagent and leucobases he interpreted at first (1937*b*) as merely showing that auto-oxidation was proceeding, but later (1940) as showing the presence of an enzyme (polyphenoloxidase) catalysing the reaction.

According to Lison (1936*b*, Chapter XVI) the subject of enzymes is one of the most confused in histochemistry. What is certain is that those enzymes that can be demonstrated cytologically catalyse the oxidation of polyphenols, polyamines, and other substances that are not themselves found in tissues; and there is no evidence whatever that such enzymes will catalyse the oxidation of other substrates. They cannot catalyse normal physiological oxidations. He adds that on the other hand one cannot say that these enzymes have nothing whatever to do with cellular respiration, because 'dans ce domaine, l'obscurité est encore totale'. Verne's evidence for the action of an enzyme (namely that the plasmal reaction is blocked by carbon monoxide and by cyanide but not by ethyl-urethane) is insufficient. It is necessary to show that these substances do not by themselves prevent the oxidation of double bonds. It is at least likely that, being themselves unsaturated, they can form addition compounds at these linkages, so protecting them from atmospheric oxygen, *in vitro*.

It seems a little unjustifiable, also, to assume that a positive result in fresh tissues is necessarily due to the formation of aldehydes by the oxidation of double bonds. So very little is known of the intermediate metabolism of fatty radicles; what is known chiefly concerns the β- or ω-oxidation of saturated acids. Smedley-MacLean (1943) considered that there is no evidence that the unsaturated acids represent stages in oxidation of fatty acids, although her view is contested by some (see the review in Hilditch, 1947). It is never possible for the histochemist to anticipate the biochemist in identifying unknown radicles in tissues unless he can prove his reagents to be specific.

The specificity of the plasmal reaction depends on the catalytic action of mercuric chloride, and the specificity of the reagents used to produce or block it, namely, phenylhydrazine and derivatives, semicarbazide and derivatives, sodium bisulphite, carbon monoxide, cyanide, dimedone, ammoniacal silver nitrate, and of course Schiff's reagent. The reaction of phenylhydrazine, semicarbazide, and their derivatives is a condensation, an intermediate addition product which is unstable being usually postulated,

$$\mathrm{R{-}CHO} + \underset{\text{phenylhydrazine}}{\mathrm{H_2NNHC_6H_5}} \longrightarrow \mathrm{RCH{=}NNHC_6H_5} + \mathrm{H_2O}$$

$$\mathrm{R{-}CHO} + \underset{\text{semicarbazide}}{\mathrm{H_2NNHCONH_2}} \longrightarrow \mathrm{RCH{=}NNHCONH_2} + \mathrm{H_2O},$$

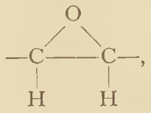
whereas the reaction with sodium bisulphite is a simple addition,

$$\mathrm{R{-}CHO} + \mathrm{NaHSO_3} \longrightarrow \mathrm{R{-}CH(OH)SO_3Na}.$$

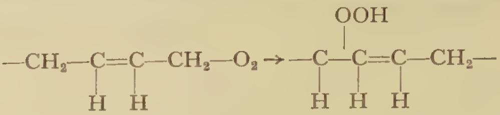
Hydrogen cyanide, always present in solutions of its salts, will react similarly,

$$\mathrm{R{-}CHO} + \mathrm{HCN} \longrightarrow \mathrm{R{-}CH(OH)CN},$$

so that the blocking of the plasmal reaction by cyanide need not require the inactivation of an enzyme. These additions are reminiscent of the additions of Schiff's reagent to double bonds, and in fact sodium bisulphite will react with some $>C=C<$ linkages (see e.g. Hickinbottom, 1948). It might be thought that the phenylhydrazine or semicarbazide condensations, which apparently require a $C=O$ linkage might be specific. Unfortunately, these compounds are also strong reducing agents and soluble to some extent in lipoids. Their presence might well prevent the continuation of oxidations in the same way as do carotenoids. The ammoniacal silver nitrate reagents react, of course, with many reducing agents, of which aldehydes are by far the most likely to occur in lipoid inclusions. Unfortunately ethylene oxide groups (Markley, 1947, p. 461) will also reduce them, and the occurrence of such groups has been claimed (e.g. by Szent-Györgyi, 1924) in the oxidation of certain acids, particularly under the influence of $-SH$ groups, which are the active groups in glutathione. It is not claimed that ethylene oxide groups, which have the constitution:



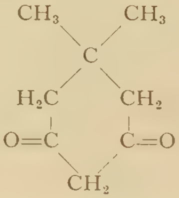
are commonly formed, but their presence must be taken into account. Research on the modes of atmospheric oxidation of unsaturated fatty acids has been carried out principally on oleic, linoleic, and linolenic acids. The very extensive and controversial literature is summarized and discussed by Hilditch (1947) and Markley (1947). Arachidonic acid has been found to occur widely in small quantities, particularly as one of the fatty acid radicles in phospholipines. Ault and Brown (1934) found that it formed over 20 per cent. of the acids in phospholipines from ox adrenal. Unfortunately no studies of its autoxidation appear to be available; as the most highly unsaturated acid present in many animal tissues, it should be of considerable importance in the plasmal reaction if Verne's theories are correct. In general, autoxidation of oleic and linoleic acids at room temperature appears to begin by the formation of a hydroperoxide group on a carbon atom next to the double link.



$$-CH_2-\underset{H}{C}=\underset{H}{C}-CH_2-O_2 \rightarrow -\underset{H}{C}-\underset{H}{\overset{OOH}{C}}=\underset{H}{C}-CH_2-$$

This is itself an oxidizer. Further stages involve the opening of the double bond to give keto and hydroxy groups, and finally the chain breaks, giving aldehydes and diacids, the aldehydes then oxidizing to acids as well. Many of the intermediate compounds are unstable and difficult to isolate, and their properties are but little known. The use of heavy metals and their salts in hastening oxidation, probably at first by destroying anti-oxidants (most likely polyphenols), is well known in industry.

If then the unsaturated acids in tissues oxidize readily, it is likely that the hydroperoxide, keto- and hydroxy-radicles, and possibly ethylene oxide groups may all coexist with double bonds. The hydroperoxides could be expected to recolour Schiff's reagent by oxidation and reduce ammoniacal silver nitrate, and to be destroyed by the reducers phenylhydrazine and semicarbazide, which could also prevent further oxidation. It is quite possible that sodium bisulphite might act in the same way, and perhaps add on to double linkages in highly unsaturated chains, as might carbon monoxide and cyanide. Dimedone, which has been used with contradictory results by Gérard and by Verne (1940) is 5 : 5 dimethyldihydroresorcinol (1 :1 dimethylcyclohexandione 3 : 5) and has the constitution:



According to Heilbron and Bunbury (1934) it is used for characterizing carbonyl compounds, in which case it should react with aldehydes and ketones, the latter being present in partly oxidized unsaturated acids. It also appears to be a reducing agent.

Recently proposed Techniques

If the oxidation could be catalysed by mercuric chloride, then all the reactions described by cytologists could be accounted for without the necessity for postulating enzymes, or the formation of aldehydes, or the presence of acetal linkages, unless the speed of the plasmal reaction could be shown to be too great to be explained by oxidation. In that case we have two possibilities: either there is a catalysing enzyme also present in tissues, or else the reaction is a true plasmal reaction in Feulgen's original sense.

This question has been investigated recently (Cain, 1949), with the result that a sharp distinction has been drawn between the true Feulgen plasmal reaction and the Feulgen–Verne reaction. The former is due, as Feulgen showed, to the release of higher aliphatic aldehydes (plasmal), presumably from acetalphosphatides, under the catalytic influence of mercuric chloride. Lengthy fixation *reduces* the reaction progressively, and finally abolishes it entirely. A control, uninfluenced by mercuric chloride, is essential. The Feulgen–Verne reaction *increases* in intensity with the length of time in fixative (or other fluids) provided that air has free access; it is not appreciably catalysed, or only slightly, with mercuric chloride; and it appears to be due to oxidation products, perhaps hydroperoxides, formed at points of unsaturation in fatty acid radicles by the action of atmospheric oxygen (or in sterols according to Shear and Kramer, 1926). The evidence brought forward by Verne that aldehydes are responsible rests on the assumptions that all the positive reactions used to prove this point are caused by a single substance in the tissues, and that the reactions used are specific for aldehydes, both of which assumptions are unjustified.

The investigations of Hayes (1947, 1949) support the conclusions reached by Cain, and this author especially emphasizes the necessity for a control section and for rapid handling of the tissues. He finds that formaldehyde-fixation 'rapidly dimishes and finally destroys' the true plasmal reaction and progressively develops a secondary reaction with Schiff's reagent that is not affected by mercuric chloride, and shows a quite different distribution from the true plasmal reaction in some tissues. He concludes that it 'probably demonstrates carbonyl-lipids other than acetals'. This is not quite in agreement with Cain's results, which suggest that other groups, equally produced by autoxidation, may be concerned, and that there is no direct evidence at all that carbonyl groups are the main cause, but the disagreement is slight. The results of these two workers, obtained completely independently, are in substantial agreement.

Hayes prescribes a technique involving the use of frozen sections, which are very inconvenient with some tissues. In Cain's technique (1949, p. 79) this difficulty is overcome by the use of very small pieces of tissue which are exposed while fresh to the action of Schiff's reagent, both with and without mercuric chloride, and then plunged into formaldehyde. The resulting formaldehyde-Schiff compound can be washed out, leaving the plasmal-Schiff compounds in place. Fixation by this method is not good, but it does allow of precise localization of the plasmal reaction within tissues. Penetration by Schiff's reagent is poor, and only very small pieces of tissue can be used.

The investigations of Danielli (1949) do not show results in accordance with those just described. The technique proposed by him (p. 68) is open to serious objection in that the fixative used (and recommended, p. 70) contained acetic acid, and that the time of fixation is given as not less than 2 hours and not more than 5 days. The use of acidic fixing fluids must be avoided in view of Feulgen and Bersin's remark (p. 413 above) that acetal linkages are destroyed in acid media. The effects of long fixation are invariably bad (Hayes, 1947, 1949; Cain, 1949). Further, it does not appear that control sections were used.

Danielli states (p. 70) that the intensity of the aldehyde reaction and the nature of its distribution in liver sections appeared to be independent of the 'physical nature of the fixative', after a considerable variety of fixatives had been tried, and that variation in the times prescribed for the stages of his technique had little effect (p. 72). This suggests, in view of the criticism just made, that it was the pseudoplasmal reaction that was observed, not the true plasmal reaction.

The supplementary tests used by Danielli to support the results of his technique are:

(i) extraction of sections with fat-solvents (to demonstrate the lipoid nature of the aldehyde);

(ii) use of azobenzene phenylhydrazine sulphonic acid, which is stated to develop a purple colour with aldehydes;

(iii) use of ammoniacal silver nitrate solution, which is reduced by aldehydes;

(iv) use of 2 : 4 dinitrophenylhydrazine, which forms a yellow hydrazone with aldehydes;

(v) treatment with hydroxylamine, before carrying out the Danielli technique for aldehydes. This, by forming an oxime with the aldehydes, prevents the formation of the aldehyde-Schiff compound.

The results of these tests are considered to prove conclusively that the colour obtained with reduced fuchsin in liver sections is due to the presence in the sections of lipoidal aldehydes. But as it has been shown (Cain, 1949) that the pseudoplasmal reaction is easily prevented by the use of reducing agents which protect unsaturated fats from atmospheric oxidation, and the reagents used in tests (ii), (iv), and (v) are reducing agents, these tests cannot be used to discriminate between a plasmal and a pseudoplasmal reaction. A positive result with test (iii) might possibly be due to the presence of other reducing agents in the tissues, and is given by ethylene oxides (Markley, 1947) which may appear in the pseudoplasmal reaction. Test (i) will of course prevent the pseudoplasmal reaction as well as the plasmal. One cannot regard this group of tests as providing support for Danielli's technique.

SUMMARY

1. The acetalphosphatides which are the precursors of the aldehydes ('plasmal') responsible for Feulgen's plasmal reaction are extremely labile compounds which hydrolyse very rapidly in acid media, and are destroyed more or less rapidly during fixation. The liberation of aldehydes from them is catalysed by mercuric chloride.

2. Ordinary unsaturated lipoids, if exposed to air, become capable of producing a colour with Schiff's reagent, which, unless a control section is

used, can be misinterpreted as a positive result for Feulgen's plasmal reaction. Mercuric chloride has little or no influence over the oxidative rancidity of unsaturated lipoids.

3. Any technique prescribed for showing the plasmal reaction must avoid acid media and prolonged fixation and handling of the tissues. A control section is a necessity.

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A Simple Method of Staining the Basement Membrane of Glomerular Capillaries

BY

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IN 1943 Thomas described a haematoxylin phosphomolybdic acid lake which stained collagen and reticulin. This observation has been confirmed and extended by the chance observation that on long standing the lake acquires the power of colouring the basement membrane of the glomerular capillaries.

METHOD

Slices of kidney were fixed in a mixture of a saturated solution of mercuric chloride (9 volumes) and commercial formalin (1 volume). After embedding in paraffin, thin sections (about 3μ) were mounted on glass, the wax was removed, and the staining was carried out in aqueous solution, as follows:

1. Lugol's iodine for 1 minute.
2. Excess of 5 per cent. sodium thiosulphate for 1 minute. Wipe the slide round the section.
3. Wash with distilled water.
4. Add the minimum amount of stain (cf. below) necessary to cover the section.
5. Wash with distilled water after 10 minutes.
6. Dehydrate, clear, and mount.

The stain was made up as follows:

Solution A.	Haematoxylin	2·5 gm.
	Dioxane	49 ml.
	Aerated water	1 ml.
Solution B.	Phosphomolybdic acid	16·5 gm.
	Distilled water	44 ml.
	Glycerol (or ethylene glycol)	11 ml.

Filter solution B. Mix equal volumes of solutions A and B and let the mixture stand for at least 10 weeks.

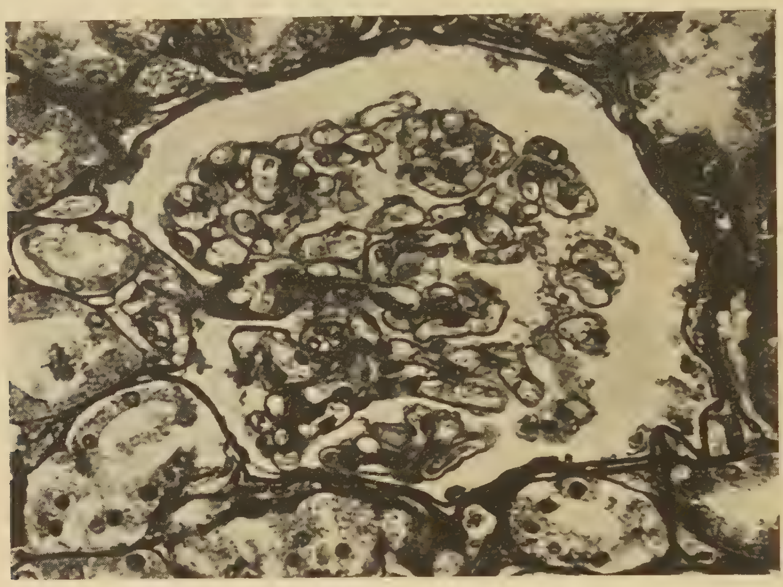
RESULTS

Collagen, reticulin, and the basement membrane of the glomerular capillaries are stained deep violet. Red blood corpuscles are purple. Nuclei are blue, but are lightly stained. The cytoplasm appears as a bluish background.

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

The basement membrane of a normal glomerulus appears as a thin, precisely delineated, structure (Text-fig. 1).

The freshly made up stain leaves the basement membrane of the glomerular capillaries uncoloured, though collagen and reticulin are stained violet, as described by Thomas (1943). It is not until the lake has ripened for 3 weeks that weak staining is observed. As ripening proceeds, the intensity of staining increases and becomes adequate in 10 weeks. A sample of the lake now 7 months old has retained its power of colouring the basement membrane of the glomerular capillaries.



TEXT-FIG. 1. Section of renal glomerulus stained to show the glomerular basement membrane. × 340. Blue-green filter.

The nature of the fixative appears to be important because attempts to utilize material fixed in 10 per cent. formol saline were unsuccessful; staining was very diffuse. If the paraffin sections affixed to the glass slides were incubated with saturated aqueous mercuric chloride for 6 days at 37° C. the depth of staining was less than in material fixed in formol mercuric chloride.

As reported by Thomas, only basic counterstains can be used, probably because of the effect of phosphomolybdic acid on cell substances. Acriflavine (1 part) in 1 per cent. acetic acid (5,000 parts) for $\frac{1}{2}$ minute provided a greenish-yellow counterstain and afforded excellent contrast.

COMMENT

This staining method is much simpler than the other well-known stains for the basement membrane of glomerular capillaries, such as the staining methods developed by McManus (1948), Lendrum, Carson, and Penny

(1945), and Heidenhain (1915). Besides, the clarity of definition of the basement membrane was much greater than when Lendrum's stain was used, or, to judge from the photomicrograph published by McManus (No. 2, p. 649), with his periodic acid-Schiff reagent stain.

SUMMARY

A method of staining the basement membrane of glomerular capillaries is described for tissue fixed in formol mercuric chloride. Ten per cent. formol saline proved to be an unsatisfactory fixative.

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Low Viscosity Nitrocellulose for Embedding Tissues

BY

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LOW viscosity nitrocellulose was introduced in the U.S.A. as a substitute for celloidin by Ruby (1933) and by Davenport and Swank (1934). It is extensively used in that country and has been recommended in text-books on technique by Bensley and Bensley (1938), Conn and Darrow (1947), and Lillie (1948). The advantages claimed are that penetration is quicker and that thinner sections can be obtained.

A similar low viscosity nitrocellulose (L.V.N.) is now obtainable in this country. We have used it by the method described by Bensley and Bensley (1938) and have found it to be most satisfactory. But as the Bensleys also showed it does suffer from certain defects. They have overcome these difficulties (personal communication, 1948) by infiltrating the tissues with L.V.N. and embedding in a tougher sample of L.V.N. with greater viscosity. Such a sample does not seem to be available here.

The defects that we noted, when using L.V.N., were that during handling, staining, and mounting of the sections, the L.V.N. tended to crack and also to break away from the tissues. The addition of a plasticizer, tricresyl phosphate, to the L.V.N. obviates these defects. Lendrum (1941) used the same plasticizer to toughen a similar type of L.V.N. for use in the Peterfi double embedding technique.

TECHNIQUE

Prepare a 20 per cent. solution of L.V.N. with tricresyl phosphate:

Absolute alcohol	210 c.c.
Ether (anaesthetic)	250 c.c.
Tricresyl phosphate	5 c.c.

Mix well and then add 140 gm. of 'Industrial Nitrocellulose damped with 7 : 3 Butyl alcohol. HX. 30/50'. This can be obtained from Imperial Chemical Industries (Paints Division), Wexham Road, Slough, Bucks., or from Hopkin & Williams Ltd., 16 St. Cross Street, Hatton Gardens, London, E.C. 1, or from E. Gurr, 108 Waterford Road, Walham Green, London, S.W. 6. The L.V.N. dissolves quickly and should be ready for use on the following day.

Prepare a 20 per cent. solution of L.V.N. similarly but omitting the tricresyl phosphate.

Prepare a 10 per cent. solution of L.V.N. by diluting the 20 per cent. solution with equal parts of a mixture of ether and absolute alcohol (equal parts).

[Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

Prepare a 5 per cent. solution of L.V.N. by diluting 1 part of the 20 per cent. solution with 3 parts of a mixture of ether and absolute alcohol (equal parts).

PROCEDURE FOR EMBEDDING TISSUES

1. Fix and dehydrate the tissues as usual.
2. Ether and absolute alcohol (equal parts)—1 day.
3. Five per cent. L.V.N.—3 to 5 days.
4. Ten per cent. L.V.N.—1 to 2 days.
5. Twenty per cent. L.V.N.—1 to 5 days.
6. Embed in the 20 per cent. L.V.N.—tricresyl phosphate solution. The paper box should be large enough to leave a margin of at least one quarter of an inch on all four sides of the tissue.
7. Allow to harden slowly in a desiccator. L.V.N. solutions harden more quickly than celloidin solutions. A small folded piece of paper under the lid of the desiccator allows enough ventilation. In 1 to 3 days the block should be adequately hard. At this stage it should be a stiff but easily deformable gel not altered in shape or size by shrinkage; it should be considerably less hard than a celloidin block is usually made. If it is allowed to harden too much or too fast the block starts to shrink and air bubbles may be forced into the block. If the block is too hard the sections will tend to roll.
8. Plunge the block into 75 per cent. alcohol. Change the alcohol at least twice over a period of 1 to 3 days. The block now becomes very hard.
9. Trim the block, removing the hard outer rim of the L.V.N. Use 20 per cent. L.V.N. solution to mount it on the wood or fibre block. Hardening is complete in a few minutes. Dip into 75 per cent. alcohol for a few more minutes.

PROCEDURE FOR CUTTING SECTIONS

Cut the sections ‘dry’. If a celloidin microtome is used, the tilt of the knife should be the same as that used for cutting celloidin. But the angle the knife makes with the direction of travel should be between 25° and 45° instead of the usual 75° used for celloidin sectioning. This prevents rolling of the sections. It is possible to cut large blocks, such as half a cat’s brain, at 15μ. Small blocks, 5×5 mm., can be cut at 5 to 7μ.

Sections can be cut at least as well on a paraffin microtome without any special modification or attachment. Spencer rotary and the small Cambridge rocking microtomes have proved satisfactory. The large Cambridge flat-cutting microtome seems to be ideally suited for this work, particularly for very thin sections of small blocks. It is comparatively easy to get 3 or 4μ sections even of hard material. The sections can be made to ribbon by coating the upper and lower surface of the block with soft paraffin.

If very thin sections of small blocks are required it may be found advantageous with some but not all tissues to omit the tricresyl phosphate from the 20 per cent. L.V.N. used for embedding. But larger blocks always cut better if the plasticizer is added.

PROCEDURE FOR HANDLING SECTIONS

1. Collect the sections in 75 per cent. alcohol; handle and stain as usual. Dyes tend to stain L.V.N. less than they do celloidin.

2. Mount the sections on to a slide from a bowl of 96 per cent. alcohol. Flatten with toilet paper moistened with the same; press the toilet paper with a glass rod and then remove it. Repeat this several times.

3. Treat similarly several times with equal parts of absolute alcohol and chloroform.

4. Treat similarly several times with the following mixture:

Xylene	2 parts
Toluene	1 part
Creosote	1 part

5. Treat similarly several times with xylene.

6. Mount in balsam.

The following alternative method is somewhat more difficult to use but gives better results with somewhat wrinkled sections of small blocks.

1. After staining, dehydrate the sections.

2. Transfer the sections to 96 per cent. alcohol.

3. Then place them in a mixture of equal parts to absolute alcohol, xylene, and chloroform.

4. Transfer single sections to a deep bowl of beechwood creosote and immediately float them on to a slide. If allowed to stay for more than 2 to 3 seconds in the bowl they will become too soft. Blot the sections on to the slide with toilet paper and smooth with a glass rod. Remove the toilet paper carefully. Immediately cover with another bit of toilet paper, smooth it and then remove it.

5. After removing as much creosote as possible, treat several times with xylene, blotting on as usual.

6. Mount in balsam.

NOTES

1. Low viscosity nitrocellulose is more explosive than celloidin and should be handled with care. When dry it would explode if hit. Exposure to direct sunlight should be avoided.

2. Slides may be coated with L.V.N. instead of celloidin. But a diluted tricresyl phosphate mixture must be used, otherwise the coating does not adhere well to the glass.

SUMMARY

Low viscosity nitrocellulose may be used as a cheap and effective substitute for celloidin. A plasticizer, tricresyl phosphate, should be added to the embedding mass. The technique for embedding, cutting, and handling the sections is described. Sections can be cut with any ordinary paraffin microtome. It is easier to use than celloidin and considerably thinner sections can be obtained.

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Wigglesworth, V. B., 1933. Quart. J. micr. Sci., **76**, 270.

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Illustration should not be redundant. Authors may be asked to provide a grant for excessive illustration. Illustrations (including lettering) should be arranged so that they may be printed as figures of a size appropriate to a page of the journal. The maximum size of a printed figure is 5″ × 7½″ = 13 cm. × 19 cm.

Illustrations should be made about half as large again as the size required in print. Thus a drawing destined to occupy a whole page should be about 7½″ × 11″ (20 cm. × 30 cm.), including lettering.

Drawings should be 'line and dot' in black process ink. (The attention of authors is drawn to 'A Method of Illustration for Zoological Papers', by H. G. Cannon, published by the Association of British Zoologists, 1936.)

Photographs should not be given if a line and dot drawing will serve the purpose. Photographic prints should be sharp and with good contrast.

Lithographic and coloured illustrations will **not** be published unless the author is prepared to make a grant covering the cost of their production.

Position of Figures and Plates should be clearly indicated by notes written in the margin of the typescript.

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Bodies of numerical data too extensive for complete publication should be summarized for discussion, and the originals deposited in the Archives established for this purpose in the British Museum (Natural History).